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14. ABSTRACT Prostate cancer almost inevitably progresses from hormone-dependent to hormone-independent state. However, the underlying mechanism is still unclear. The purpose of this study is to test the hypothesis that aberrant chromatin modification plays a critical role in prostate cancer progression. We proposed to analyze histone modifications on AR target genes such as PSA and identify the responsible enzymatic activities in prostate cancer cells. During the entire project time, we have made several major findings. First, we found that, in androgen-dependent cells, the level of histone acetylation at PSA gene is regulated by androgen and anti-androgen, regulatory DNA elements and promoter context. Secondly, we found that, in androgen-independent cells, histones are hyperacetylated without hormone stimulation, and AR and the coactivators containing histone acetylase (HAT) activities are recruited in an androgen-independent manner. Thirdly, we found that depletion of the HAT coactivator ACTR effectively blocks both androgen-dependent and -independent prostate cancer cell proliferation. Together, these results suggest that HAT proteins play an important role in the progression to hormone-refractory state. Interestingly, we also found that, in androgen-independent cells, AR and the coactivator ACTR controls cancer cell proliferation through distinct mechanisms. The findings support the idea that targeting HAT proteins such as ACTR can be an effective way to stop prostate cancer progression to androgen independence and the growth of hormone-refractory prostate cancer.					
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INTRODUCTION

The progression of prostate cancer (CaP) from hormone-dependent to hormone-refractory state represents one of the major hurdles in the successful treatment of cancer patients. Early studies showed that nearly all cancers retain androgen receptor (AR)-mediated signaling pathway. One of the working models has been that shifts from paracrine to autocrine expression of growth factors and abnormal function of their receptors contributes to the progression to androgen-independent cancer through modulating the function of AR. We proposed a new hypothesis that chromatin histone modification and remodeling could be a key step in CaP progression. We proposed to identify altered histone modification patterns linked to progression of prostate cancer and the enzymatic activities that are responsible for the alteration. The cope of this study was to first analyze histone acetylation and phosphorylation patterns on androgen receptor (AR) target genes such as PSA in prostate cancer cells that can be used as models for different stages of CaP. Comparison of the patterns of histone modifications between these cells will allow us to identify the chromatin modifications and responsible enzymes that are altered during the progression of prostate cancer.

BODY

Task 1. Identify abnormal histone acetylation and phosphorylation at important androgen receptor target genes and other genes associated with prostate cancer progression (months 1-18):

- As described in our first report, we found that DHT stimulates histone hyperacetylation at both the enhancer and promoter elements of PSA gene in androgen-dependent LNCaP cells. Later described in our second report, we found that, in androgen-independent LNCaP derivative C4-2B cells, chromatin histones at the PSA gene regulatory region are constitutively hyperacetylated, and that DHT treatment of C4-2B cells has much reduced effect on histone acetylation than original LNCaP (Fig 1).

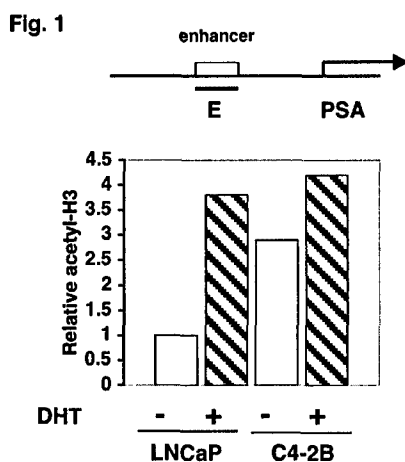


Fig. 1. Chromatin histone (H3) acetylation at the enhancer region of PSA gene in LNCaP and its derivative C4-2B was analyzed by ChIP assay. Cells (2×10^7) were maintained in hormone-deprived medium for 3 days, and then treated with 10^{-9} M DHT for 6 hours, before being harvested for ChIP assay. Precipitated genomic DNA was analyzed for enrichment of specific sequence at the PSA enhancer region (the E amplicon) by semi-quantitative PCR, as described in Fig 1 of the Appendix 1. Digital images of the PCR products were quantified. The obtained values were plotted as relative level of H3 acetylation with the value obtained from LNCaP cells in the absence of hormone set as 1.

- In our second report, we described the finding that a high level of androgen-independent assembly of RNA polymerase II at the PSA promoter in C4-2B cells. Since then, we were trying to determine whether this hormone-independent recruitment of Polymerase II is mediated by AR and HAT proteins such as ACTR/AIB1. We now report that indeed AR is required for the hormone-independent Polymerase II recruitment and the transcription of PSA gene. This is based on our data that RNAi-mediated AR depletion in C4-2B cells decreased PSA gene expression and Polymerase II recruitment at the PSA gene promoter. However, knocking down of ACTR did not show any significant effect (Fig 2). This is likely due to the functional redundancy among the three members of the p160/SRC family of coactivators expressed at high levels in the cells.

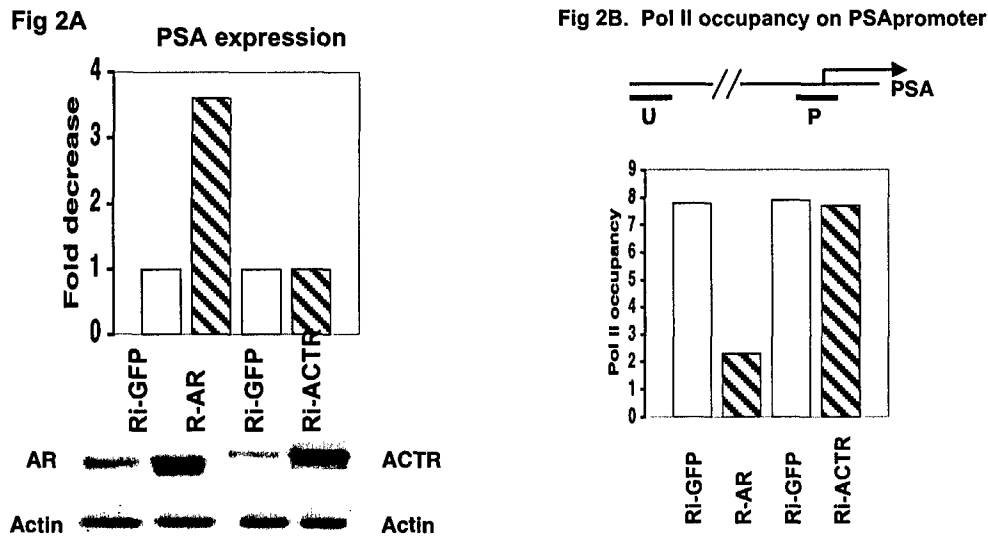


Fig. 2. Knockdown of AR, not ACTR, affects PSA gene transcription. LNCaP cells were infected with adenovirus vectors for RNAi-mediated silencing of AR or ACTR expression, or the control Ad-Ri-GFP, as described in Fig 1 of Appendix 2. Two days later, cells were treated with 1 nM DHT, and after another 24 hours harvested for RNA and protein analysis (Fig 2A, top and bottom panels respectively). Real-time PCR was performed for assessing PSA expression. Fold decrease in DHT-stimulated PSA expression was presented with the data of PSA real-time PCR in cells treated with Ad-Ri-GFP set as 1. Cells treated similarly with the adeno-RNAi and DHT were also harvested for ChIP assay with anti-RNA polymerase II antibody (Fig 2B), as described in Appendix 1. The relative Pol II occupancy at PSA promoter was calculated by dividing the PCR product signals from the P amplicon with that from an upstream (U) region (about 8 kb away from the transcription initiation site of PSA).

- In our second report, we described that we had examined whether histone phosphorylation plays a role in AR-mediated control of gene expression. Using the antibody generated against phosphorylated histone H3 (from either Upstate Biotech or Cell Signaling), we detected a low level of histone phosphorylation at the PSA enhancer region (about 2 fold higher at the E amplicon than the U amplicon which we considered to be the background) in LNCaP and C4-2B cells cultured in the presence or absence of 1 nM DHT. Because the difference is

- marginal, we were not confident to conclude that histone phosphorylation might play a significant role in AR-mediated control of PSA gene expression. Since then, we have examined more AR target genes such as KLK2 and obtained similar inconclusive evidence.
- Also discussed in our second report, we intended to ectopically express AR in the immortalized but non-malignant human prostate epithelial cell line (RWPE-1) obtained from Dr. Mukta M. Webber at Michigan State University, in order to identify the difference in chromatin modification between non-malignant and cancer prostate epithelial cells. Here, we report that although we were able to express AR in RWPE-1 cells using adeno-vector, the AR-expressing RWPE-1 cells do not respond to androgen in proliferation (Fig 3). Furthermore, we could not detect, by either RT-PCR or Western analysis, PSA gene expression in either the AR-expressing RWPE cells (Fig 4b), or the original RWPE-1 cells (data not shown). These results precludes us from pursuing the approach by comparison between non-malignant and cancer prostate cells.

Fig 3. MTT cell proliferation assay of AR-expressing RWPE cells

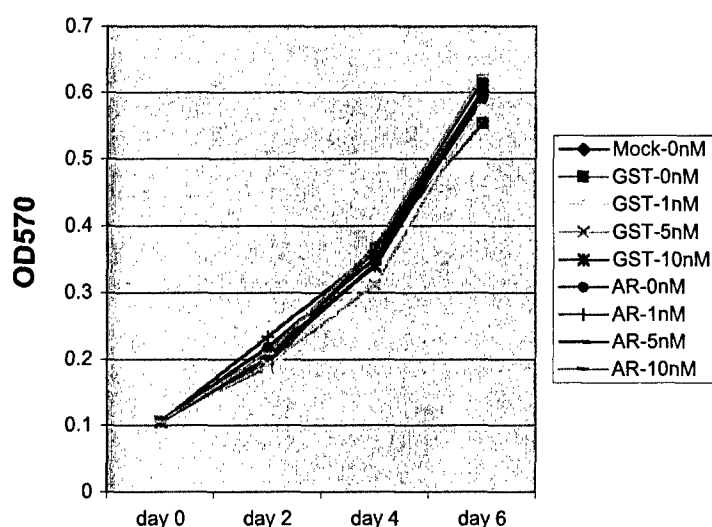
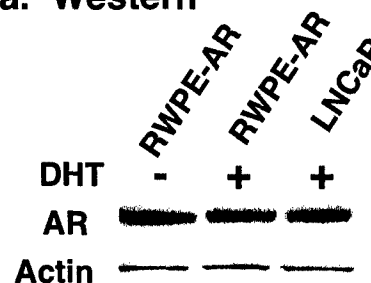


Fig 4. Analysis of PSA expression in AR-expressing RWPE cells

a. Western



b. RT-PCR



Fig 3. RWPE-1 cells were plated in 96-well plates, infected with recombinant adenovirus vectors expressing AR or GFP, or mock infected, then treated with or without 1×10^{-9} M DHT. Different days after infection, cell proliferation was examined by MTT assay.

Fig 4. RWPE-1 cells were plated in 6-well plates, infected with the AR-adeno-vector, and treated with or without 1×10^{-9} M DHT. One day after DHT treatment, cells were harvested for Western (top panel), or RT-PCR analysis of PSA expression (bottom panel). LNCaP cells treated with DHT were analyzed for AR and PSA expression for comparison.

Task 2. Determine the role of specific histone acetyltransferases (HATs), histone deacetylases (HDACs) and the histone kinases in prostate cancer progression (months 18-36):

- In our second report, we observed that DHT induced the recruitment/occupancy of nuclear receptor coactivators including three members of the p160/SRC-1 family (ACTR, TIF1 and SRC1) and CBP/p300 (Figure 1 of Appendix 1). Both the p160 coactivators such as ACTR and SRC-1 and CBP/p300 proteins are demonstrated to possess histone acetylase (HAT) activities. We also found that RNA polymerase II is directly recruited to the PSA enhancer on androgen stimulation (Fig 2 of Appendix 1). Strikingly, we found that the HAT protein ACTR is required for the direct recruitment of Pol II complex to the PSA enhancer induced by androgen (Fig 2 of Appendix 1). Significantly, we found that elevated levels of ACTR enhance both androgen-dependent and -independent PSA expression. This activity of ACTR correlates with its ability to mediate the recruitment of Pol II directly to the PSA enhancer (Fig 5 of Appendix 1).
- Also in second report, we found that low concentrations of flavopiridol, a Cdk inhibitor, selectively inhibit androgen induction of PSA expression, and strongly block the assembly of Pol II complex at the promoter without affecting its recruitment to the enhancer. These results suggest that cyclin-dependent kinase activity is required for DHT-stimulated transfer of RNA polymerase II from the PSA enhancer to promoter in the process of induction of PSA transcription (Fig 4 of Appendix 1).
- Here we report that high levels of coactivator ACTR, but not TIF2, are required for the proliferation of LNCaP and its derivative C4-2B cells (Fig 3 of Appendix 2). Interestingly, depletion of AR and ACTR affects the expression of distinct cell cycle genes. As shown in Fig 4A and 4B of Appendix 2, knocking down AR or ACTR in LNCaP cells resulted in a decrease of E2F1 and cyclin D1 protein level. Interestingly, knocking down AR, but not ACTR, significantly inhibited the expression of cdc2 and cdk2 proteins. Conversely, knocking down ACTR, but not AR, decreased the expression of cyclin A and cyclin B. As shown in Fig 4C and 4D of Appendix 2, in C4-2B cells, while both AR and ACTR reduction led to decreased expression of E2F1 and cyclin A, knocking down AR, but not ACTR, also significantly inhibited the expression of cyclin B, cdc2 and cyclin D1 proteins; and knocking down ACTR, but not AR, also decreased the expression of cyclin E and cdk2. Furthermore, the results of quantitative RT-PCR analysis with C4-2B cells shown in Fig 5 of Appendix 2 indicated that the differential effects of AR and ACTR depletion is largely mediated at the mRNA level for cyclin E, cyclin B, cdc2 and cdk2, as >2-fold decrease of their transcripts can be observed with cells treated with the corresponding Ad-RNAi vectors. These results suggest that the coactivators may control hormone-refractory prostate cancer growth through mechanisms that are both overlapping and distinct from that of AR. These results are included in a manuscript that has been submitted to Cancer Research.
- As reported previously, we initiated the study on the role of histone deacetylases (HDACs) in androgen control of gene expression and in prostate cancer progression. We observed that low levels of HDAC1 and HDAC2 occupy the promoter and enhancer of AR target gene PSA, and that anti-androgens such as casodex could strongly enhance the recruitment of these histone deacetylases. We tried to determine whether HDAC1 and HDAC2 play a role in androgen induction of gene expression and control of CaP cell proliferation by RNAi-mediated depletion. Our preliminary results indicated that knockdown of HDAC1 or HDAC2 in C4-2B cells did not appear to affect PSA gene expression and cell proliferation. We have

also tried to detect by ChIP assay the involvement of several candidate histone kinases including p38 MAP kinase and MSK1 in PSA gene expression. Until this point, we have not been successful. Combined with the lack of significant histone phosphorylation detected on PSA gene, these results suggest that histone phosphorylation may not play a critical role in androgen induction of PSA gene expression.

RESEARCH ACCOMPLISHMENTS (June 2001-December 2004 including the 6 months of no cost extension)

During the entire project time, we have made the following major findings.

- In androgen-dependent cells, DHT induced marked histone hyperacetylation at both the promoter and the enhancer of PSA gene, while anti-androgens such as casodex induced histone hypoacetylation primarily at the enhancer elements. These results suggest that hormones, regulatory DNA elements and promoter context control AR to form complexes that possess distinct chromatin modification activities.
- In androgen-independent cells, histones are hyperacetylated without hormone stimulation, and AR and the coactivators containing histone acetylase (HAT) activities are recruited in an androgen-independent manner at both the enhancer and promoter of PSA.
- Depletion of the HAT coactivator ACTR by RNAi effectively blocks both androgen-dependent and -independent prostate cancer cell proliferation. Together, these results suggest that HAT proteins play an important role in the progression of hormone-dependent prostate cancer to hormone-refractory state.
- Interestingly, in androgen-independent cells, AR and the coactivator ACTR regulate the expression of different genes that are involved in control of cell cycle, suggesting that distinct mechanisms evolves during progression to androgen independence for AR and other key players to control prostate tumor growth.

REPORTABLE OUTCOMES

Publication:

Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ, Chen HW. Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *Proc Natl Acad Sci U S A*. 2003. 100(5):2226-30.

Manuscript submitted to Cancer Research:

Zou JX, Zhong ZY, Shi X-B, Tepper C, deVere White RW, Kung HJ, Chen HW. ACTR/SRC-3 and androgen receptor control prostate cancer cell proliferation and tumor growth via distinct mechanisms.

Presentation by Chen HW at Keystone Symposium "Hormonal Regulation of Tumorigenesis", February 20-25, 2005, Monterey, CA, USA

AR and the p160/SRC coactivators control the proliferation of androgen-independent prostate cancer cells via distinct mechanisms.

Presentation by Zou JX at the 96th Annual Meeting of AACR, April 16-20, 2005. Anaheim, CA, USA.

Members of the p160/SRC coactivators and androgen receptor control prostate cancer cell proliferation through regulating distinct cell cycle gene expression.

Degrees obtained that were partly supported by this award:
PhD to Maggie C. Louie, November, 2004.

CONCLUSIONS

The findings described above indicate that histone acetylation is indeed involved in AR-mediated control of gene expression and that several of the known HAT proteins are likely responsible for the control of ligand-induced histone acetylation at PSA gene. More significantly, our demonstration that high levels of HAT protein ACTR are required for both androgen-dependent and -independent prostate cancer cell proliferation suggests a critical role for ACTR in the progression of CaP to androgen independence. Given the fact that nuclear receptor coactivators such as ACTR is overexpressed in later stages of prostate cancer (Gregory et al. 2001; Gnanapragasam et al., 2001; AgoulNIK et al., 2005; Zhou et al., 2005), the findings summarized above support the idea that targeting HAT proteins such as ACTR can be an effective way to stop prostate cancer progression to androgen independence and the growth of hormone-refractory prostate cancer.

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Gnanapragasam VJ, Leung HY, Pulimood AS, Neal DE, Robson CN. 2001. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. *Br J Cancer*. **85**(12):1928-36.

AgoulNIK, I. U., Vaid, A., Bingman, W. E., 3rd, Erdem, H., Frolov, A., Smith, C. L., Ayala, G., Ittmann, M. M., and Weigel, N. L. Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. *Cancer Res*, **65**: 7959-7967, 2005.

Zhou, H. J., Yan, J., Luo, W., Ayala, G., Lin, S. H., Erdem, H., Ittmann, M., Tsai, S. Y., and Tsai, M. J. SRC-3 is required for prostate cancer cell proliferation and survival. *Cancer Res*, **65**: 7976-7983, 2005.

***APPENDICES/publications:**

1. Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ, Chen HW. Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A. 2003. 100(5):2226-30.
2. Zou JX, Zhong ZY, Shi X-B, Tepper C, deVere White RW, Kung HJ, Chen HW. ACTR/SRC-3 and androgen receptor control prostate cancer cell proliferation and tumor growth via distinct mechanisms. (manuscript submitted to Cancer Research)

Personnel (not salaries) receiving pay from the research effort:

Hongwu Chen
Maggie Louie
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Zhengyu Zhong

Androgen-induced recruitment of RNA polymerase II to a nuclear receptor–p160 coactivator complex

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Communicated by Ronald M. Evans, The Salk Institute for Biological Studies, San Diego, CA, December 23, 2002 (received for review August 27, 2002)

The androgen receptor, like other nuclear receptors, activates target genes by binding to hormone-responsive enhancers. Here we demonstrate that androgen induces robust recruitment of androgen receptor, members of the p160 coactivator family, and CREB-binding protein/p300 specifically at the distant enhancer of prostate-specific antigen (PSA) gene. Unexpectedly, we found that RNA polymerase II (Pol II) is directly recruited to the enhancer in a hormone-dependent manner, independent of the proximal promoter, and that the isolated PSA enhancer can mediate efficient androgen induction of transcription. Inhibition of the Pol II carboxyl-terminal domain kinase activity with low concentrations of flavopiridol blocks Pol II transfer from the enhancer to the promoter and selectively abolishes PSA induction by androgen. Moreover, elevated levels of the p160 coactivator ACTR/AIB1 increase both androgen-dependent and -independent PSA expression, by facilitating Pol II recruitment to the enhancer. These results support a model in which nuclear receptors and their coactivators mediate hormone induction by serving as a staging platform for Pol II recruitment.

Androgen is a key regulator of cell growth and differentiation in male sexual development and function as well as in the progression of prostate cancer. These hormonal effects are mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily, which consists of ligand-dependent transcription factors (1). Recently, a growing number of nuclear proteins have been found to associate with AR and are postulated to mediate transcriptional control by the receptor (2, 3). Among them are members of the p160 coactivator family, including SRC-1, TIF2/GRIPI, and ACTR (AIB1/RAC3/TRAM1/pCIP). The p160 coactivators associate with nuclear receptors in a hormone-dependent fashion primarily through the central receptor–interaction domain that harbors several LXXLL motifs.

Although p160 coactivators possess intrinsic histone acetylase (HAT) activities, they are also capable of recruiting other HAT proteins such as CREB-binding protein (CBP), p300, and PCAF (4, 5), and the nuclear protein arginine methyltransferases CARM1 and PRMT (6, 7). Both the acetylases and methylases can regulate transcription by modifying nucleosomal and non-nucleosomal nuclear proteins (7, 8). However, how the p160 coactivators promote RNA polymerase II (Pol II) transcription is still not well understood. Domain mapping experiments suggest that transactivation by the p160 coactivators is largely attributable to their recruitment of CBP/p300 and the HAT activities (4, 9–12). Although earlier experiments indicate that CBP/p300 may interact directly with the Pol II complex (13, 14), this notion lost currency with the discovery of the importance of the HAT activity in transcriptional activation and the more recent demonstration that the simple recruitment and assembly of Pol II machinery in mammalian cells is not sufficient for productive transcription (15, 16).

Previously, we and others demonstrated that hormone induces histone hyperacetylation at the proximal promoters of nuclear receptor target genes presumably through the recruitment of p160 and p300/CBP coactivator complexes (8, 17). To further understand the molecular underpinnings of hormone induction

of gene expression, we analyzed androgen-induced recruitment of AR and its associated coactivators to the entire 5' regulatory region of the prostate-specific antigen (PSA) gene in hormone-responsive LNCaP cells. High-level androgen induction of PSA expression is conferred by an enhancer centered at ≈ -4.2 kb on the PSA gene, which contains multiple tandem androgen response elements (18–20). Here, we show that on androgen stimulation, the p160 and CBP/p300 coactivators are preferentially recruited to the enhancer. This observation has led to our unexpected finding that Pol II is directly recruited to the enhancer in an androgen-dependent manner and that Pol II carboxyl-terminal domain (CTD) kinase activities are critical for the possible tracking of Pol II from enhancer to promoter.

Materials and Methods

Chromatin Protein Association Assays. Chromatin immunoprecipitation (ChIP) assay was performed with LNCaP cells cultured in hormone-depleted medium for at least 3 days before treatment with ligands. *In vitro* protein association assay with immobilized reconstituted chromatin and details of the ChIP procedures and materials used are provided in *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org.

Transient Transfection. LNCaP cells were transfected with luciferase reporter pGL3-basic, or its derivatives with PSA upstream sequences, pRL-SV40 *Renilla* luciferase, along with pCMX-ACTR or pSh-CMV-TIF2, by using the Lipofectin reagent. Cells were then treated with 10 nM 5 α -dihydrotestosterone (DHT) for 24 h before harvest for luciferase assay. The pGL3-PSA reporters were constructed by inserting restriction fragments of the PSA upstream sequences with the indicated lengths as follows: 5.85-kb entire PSA upstream sequences, *HindIII* to *HindIII*; 630-bp PSA proximal promoter sequences, *EcoRI* to *HindIII*; 1.5-kb sequence containing the PSA enhancer, *XbaI* to *BamHI*; and 2.0-kb fragment containing the PSA enhancer and an immediate downstream sequence, *XbaI* to *StuI*.

Recombinant Adenovirus Vectors and Gene Expression Analysis. ACTR cDNA was inserted into a modified pShuttle-CMV vector with 3 \times hemagglutinin tag sequence at the C terminus. The resulting constructs were used to generate recombinant adenovirus as described (8). Viruses were purified by centrifugation in CsCl step gradients. Viral titers were determined by end-point cytopathic effect assay with GFP adeno-vector as a reference. Gene expression was compared by using RT-PCR. Total RNA was prepared with the TRIzol reagent, and the cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase. Gene-specific primers for the PCR are provided in *Supporting Text*.

Abbreviations: AR, androgen receptor; PSA, prostate-specific antigen; Pol II, RNA polymerase II; CTD, carboxyl-terminal domain; ChIP, chromatin immunoprecipitation; DHT, 5 α -dihydrotestosterone; CBP, CREB-binding protein.

[†]M.C.L. and H.Q.Y. contributed equally to this work.

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Results

AR and p160-CBP/p300 Coactivators Are Preferentially Recruited to the PSA Enhancer on Androgen Stimulation. To investigate the role of AR-associated coactivators in PSA induction, we first examined androgen-induced, endogenous AR and coactivator occupancy over the entire 6-kb upstream regulatory region of PSA in LNCaP cells by ChIP assay. For semiquantitative measurement of the precipitated DNA, we used a panel of PCR primer pairs with approximately equal efficiency to amplify partially overlapping fragments (≈ 300 –500 bp) encompassing the 6-kb region (Fig. 1*A Top*). The enhancer sequence was amplified as fragments D, E, and F, whereas the sequence of proximal AREs and initiation site was covered by fragments O and P. ChIP assay with anti-AR antibody demonstrated clearly that, on androgen induction, AR was strongly recruited to the enhancer region and this AR occupancy persisted hours after DHT addition (Fig. 1*A*, α -AR). Interestingly, the association of AR with the proximal promoter was only marginal after DHT stimulation (Fig. 1*A* and Fig. 6, which is published as supporting information on the PNAS web site). In the same experiments, we also measured the recruitment of p160 coactivators and coregulators CBP/p300. Indeed, robust recruitment of ACTR, TIF2, and CBP/p300 to the PSA 5' regulatory region was observed when cells were treated with DHT. Importantly, as seen for AR, androgen-induced coactivator recruitment was highly restricted to the enhancer (Fig. 1*A*). Next, we transfected LNCaP cells with two reporters containing either the 6-kb upstream sequence or the 630-bp proximal sequence of PSA. Results in Fig. 1*B* indicate that the enhancer-containing 6-kb upstream sequence, not the proximal promoter, was capable of mediating androgen induction. In agreement with the ChIP results, cotransfection of coactivator ACTR and TIF2 constructs further potentiated androgen-stimulated transcription through the 6-kb sequence, but not the 630-bp promoter (Fig. 1*B*). Taken together, these results suggest that the androgen-responsive enhancer of the PSA gene mediates transcriptional activation through direct recruitment of AR and the p160-CBP/p300 coactivator complex.

Pol II Is Directly Recruited at PSA Enhancer, Independent of the Proximal Promoter, on Androgen Induction. Transcription factors and their coregulators are believed to regulate transcription, in part, by controlling the recruitment and/or assembly of the general transcription machinery (16). We, therefore, examined how Pol II is recruited to the upstream sequence of the PSA gene in response to androgen stimulation. In agreement with our previous results that hormones induce Pol II recruitment to the promoters of estrogen receptor target genes (8), significant recruitment of Pol II to the PSA promoter region is observed on androgen induction (Fig. 2*A*, open bars). Intriguingly, strong androgen-dependent Pol II cross-linking to the enhancer was also detected. In fact, the DHT-induced Pol II cross-linking at the enhancer is more abundant than that at the promoter (Fig. 2*A*, filled bars). This unexpected result prompted us to extend the analysis to the other region of the PSA regulatory sequence. As shown in Fig. 2*B*, remarkably, a significant level of Pol II cross-linking was observed over the region between enhancer and promoter. However, when regions further upstream were analyzed, little Pol II occupancy was detected (Fig. 7, which is published as supporting information on the PNAS web site).

An attractive model of how an enhancer influences the function of the transcription machinery involves the physical interactions of protein complexes assembled at the enhancer with those at the promoter, leaving a DNA loop in between (i.e., the looping model). Formally, the cross-linking of Pol II to the enhancer can be attributed either by the contact of protein complexes formed at the enhancer with Pol II assembled at the promoter or by the occupancy of Pol II at the enhancer

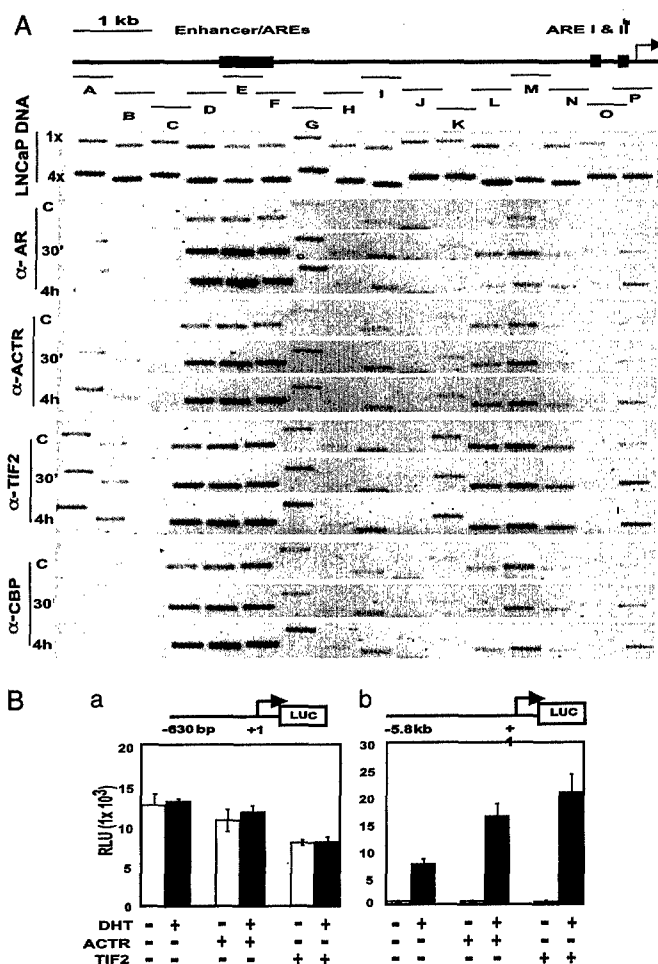


Fig. 1. AR and p160-CBP/p300 coactivators are preferentially recruited to the PSA enhancer to mediate androgen activation of PSA promoter in LNCaP cells. (A) DHT induces the recruitment of AR and p160-CBP/p300 coactivators preferentially at the PSA enhancer. A diagram of the 6-kb upstream regulatory region of AR target gene PSA with androgen-responsive enhancer and elements indicated above and the various fragments amplified in the ChIP assay marked below as A to P. The amplification efficiency of the primer pairs for the different fragments was assessed by PCR under the same cycling condition by using two different volumes (1 \times and 4 \times) of diluted LNCaP cell genomic DNA prepared during the ChIP procedures. Indicated antibodies were used to perform ChIP assay with LNCaP cells treated with 10 nM DHT for 30 min or 4 h, or mock-treated (C) for 4 h, to measure chromatin occupancy of AR, ACTR, TIF2, and CBP along the 6-kb sequence of PSA. Input chromatin DNA taken before immunoprecipitation from different samples was analyzed by PCR and found to be identical (data not shown). (B) LNCaP cell transfection was performed to analyze the ability of PSA enhancer and/or promoter to mediate androgen transcriptional induction and coactivation function. LNCaP cells in hormone-depleted media were cotransfected with either the 630-bp PSA proximal promoter-Luc reporter (a) or the 5.85-kb entire PSA upstream regulatory sequence-Luc (b) and the ACTR or TIF2 expression plasmids. Transfected cells were then treated with 10 nM DHT (filled bars) or mock-treated (open bars) for 24 h before measuring luciferase activities.

independent of promoter (Fig. 2*C*). The first scenario would predict an efficient cross-linking of AR and p160-CBP/p300 complex to the promoter sequence, which was not observed in our analysis (Fig. 1). To further discern the two possibilities, we took advantage of our observation that AR is recruited primarily to the enhancer. If Pol II is directly recruited to the enhancer, it would physically co-occupy the enhancer with AR. Therefore, fragmented chromatin-protein adducts precipitated by anti-Pol II antibody should contain the enhancer chromatin fragment

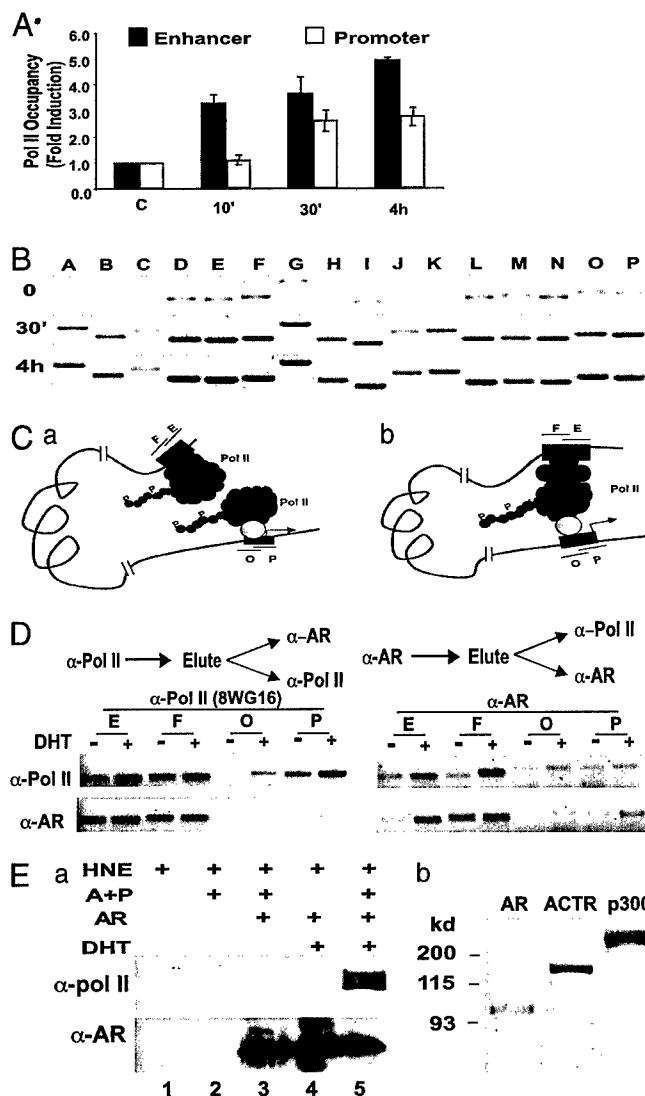


Fig. 2. Pol II is directly recruited to the PSA enhancer on androgen stimulation. (A) ChIP assay was performed with α -Pol II antibody (N-20) to analyze the Pol II occupancy at the enhancer (fragment E, filled bars) or the promoter (fragment P, open bars) of the PSA gene. The relative amount of Pol II occupancy in the presence versus absence of 10 nM DHT (fold induction) was determined by quantifying the PCR products obtained from three experiments. (B) Pol II occupancy over the entire PSA upstream regulatory sequence was analyzed by using ChIP assay as in A with the monoclonal α -Pol II antibody 8WG16. (C) Two possible modes of androgen-induced Pol II occupancy at the PSA 5' regulatory region: Pol II is recruited to the enhancer and promoter independently (Left) or Pol II is recruited to the promoter and physically interacts with protein complex formed at the enhancer (Right). (D) Sequential ChIP assay was used to analyze the association of Pol II and AR at the PSA promoter (O and P) or the enhancer (E and F). LNCaP cells were treated with 10 nM DHT (+) or mock-treated (-) for 1 h before ChIP assay. Chromatin fragments were first immunoprecipitated with α -Pol II (Left) or α -AR antibody (Right). Immunocomplexes were eluted from the agarose beads and diluted for a second immunoprecipitation for AR or Pol II occupancy analysis. (Ea) Immobilized nucleosomal PSA enhancer fragment (-4500 to -3750) was incubated with HeLa nuclear extract (HNE), in the presence or absence of purified AR, ACTR and p300 (A+P) proteins shown in b, and DHT as indicated, followed by extensive washes and Western blotting with indicated antibodies. (b) Coomassie staining of purified AR (100 ng), ACTR (500 ng), and p300 (500 ng) proteins.

that is also cross-linked with AR. As shown in Fig. 2D Left, when the eluate of anti-Pol II precipitates were reprecipitated with anti-AR antibody, indeed, the second precipitates contained the

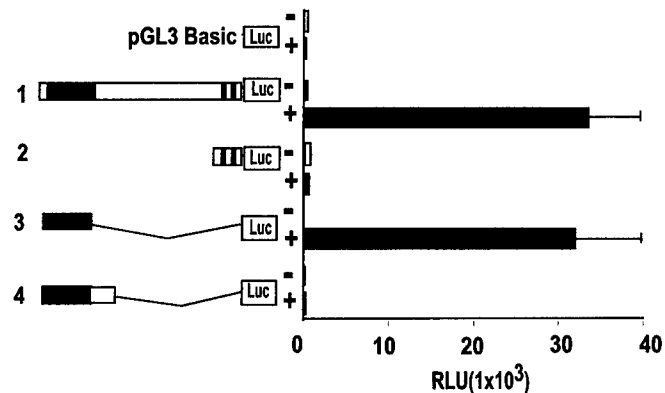


Fig. 3. The PSA enhancer, in isolation, mediates androgen induction of transcription independent of PSA proximal promoter. Transient transfection was performed as in Fig. 1B with the promoterless pGL3 basic-Luciferase reporter, the 5.85-kb PSA upstream regulatory sequence linked to the Luc (lane 1), the 630-bp PSA proximal promoter-Luc (lane 2), the 1.5-kb sequence containing the PSA enhancer-Luc (lane 3), or the 2.0-kb fragment containing the PSA enhancer and immediate downstream sequence (lane 4). Transfected cells were treated with 10 nM DHT (filled bars) or mock-treated (open bars) for 24 h before measuring luciferase activities. RLU, relative luciferase unit.

enhancer DNA. In contrast, no significant amount of promoter sequence can be detected, in accordance with the low occupancy of AR at promoter. When the same eluate of Pol II precipitates was reprecipitated with anti-Pol II antibody, strong hormone-dependent cross-linkings were seen at both enhancer and promoter, indicating that the failure to detect promoter sequence in the anti-AR immunoprecipitation is not caused by the preferential loss of promoter chromatin fragment during the elution. Again, consistent with the notion that AR and Pol II co-occupy enhancer but not promoter, a sequence from the enhancer but not promoter was detected after the anti-AR precipitates were subsequently immunoprecipitated with the Pol II antibody (Fig. 2D Right).

To determine whether the occupancy of Pol II at the enhancer is independent of the promoter sequence of PSA, we incubated the PSA enhancer chromatin assembled *in vitro* and immobilized on beads with HeLa nuclear extract in the presence or absence of purified AR, ACTR, and p300 proteins. After extensive washing, proteins associated with the chromatin were detected by Western blotting. Results in Fig. 2E show that both phosphorylated and unphosphorylated Pol II (top and bottom bands, respectively, in lane 5) was recruited to the enhancer only in the presence of DHT, and the AR, ACTR, and p300 proteins. Interestingly, AR is recruited to the enhancer independent of DHT. Taken together, these results indicate that, on androgen stimulation, Pol II is directly recruited to the enhancer, independent of the PSA promoter.

The Isolated Enhancer Mediates Strong Androgen Induction of Transcription Independent of PSA Proximal Promoter. Our observation that Pol II is directly recruited to the PSA enhancer on androgen induction suggests that Pol II might function to initiate transcription through the enhancer. To test this idea, we performed transfection assay with promoterless reporter constructs containing different PSA 5' upstream sequences. Remarkably, when transfected into LNCaP cells, the reporter linked to a PSA 5' fragment containing essentially the core enhancer sequence (construct 3) mediated a robust DHT-induced reporter gene activity similar to the reporter that contains the entire 5' PSA regulatory sequences (construct 1, Fig. 3). Surprisingly, however, a similar reporter containing 500-bp extra sequences downstream of the enhancer (construct 4) was completely inactive in

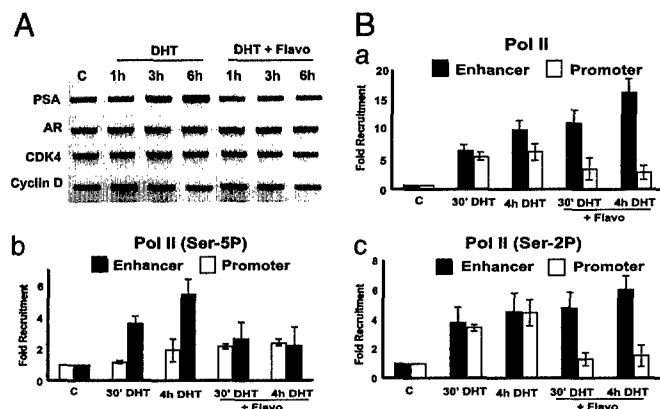


Fig. 4. CTD kinases are involved in possible Pol II transfer from enhancer to promoter and androgen induction of PSA. (A) Flavopiridol selectively blocks DHT induction of PSA expression measured by RT-PCR. RNA was isolated from LNCaP cells treated with either 10 nM DHT or DHT plus 100 nM flavopiridol (DHT+Flavo) for the indicated times. (B) Flavopiridol blocks possible Pol II transfer from enhancer to promoter. LNCaP cells were treated as in A for the indicated times. ChIP assay using α -Pol II 8WG16 (recognizes nonphosphorylated and phosphorylated Pol II), H5, and H14 (recognize Pol II CTD phosphorylated at Ser-2 and Ser-5, respectively) was performed as in Fig. 2A to analyze the occupancy of the different phosphorylated forms of Pol II at the enhancer and the promoter.

response to DHT induction, suggesting that at least part of the enhancer function is controlled by an enhancer-context-dependent mechanism. By Northern analysis with a 1.5-kb fragment containing the enhancer sequence as a probe, we were unable to detect any mature transcripts initiated around the enhancer region in DHT-treated LNCaP cells (data not shown), indicating that the enhancer does not serve as a gene promoter in its natural chromatin context. Nevertheless, these results suggest that the androgen-responsive enhancer of the PSA gene not only can independently recruit Pol II, but also has the potential to mediate transcription.

Pol II CTD Phosphorylation Is Important in Mediating Pol II Transfer from the Enhancer to Promoter. Phosphorylation of Pol II CTD at serines 5 and 2 of the heptad repeats is important in multiple stages of transcription, particularly the transition of Pol II function from promoter arrest to elongation (21). We thus examined whether Pol II-CTD phosphorylation could play a role in the conversion of Pol II recruited at the enhancer to the one functioning at the promoter. To this end, we resorted to the CTD kinases inhibitor flavopiridol (22, 23). We first analyzed the effect of flavopiridol on androgen-induced PSA expression by Pol II. Consistent with the previous reports that low concentration (<200 nM) of flavopiridol does not show inhibitory effect on most gene transcription (23), expression of AR itself and cell cycle genes such as Cdk 4 and cyclin D was not significantly affected by the treatment with 100 nM flavopiridol. In contrast, under the same condition, DHT-induced PSA transcription was completely blocked (Fig. 4A). We then examined the effect of flavopiridol treatment on androgen-induced Pol II recruitment/occupancy at PSA gene (Fig. 4B). Strikingly, treating cells simultaneously with DHT and flavopiridol strongly inhibited androgen-induced Pol II recruitment at the promoter while increasing the Pol II occupancy at the enhancer. Further analyses with antibodies recognizing Pol II phosphorylated at CTD Ser-2 and Ser-5 indicate that the decrease of recruitment in Ser-2 phosphorylated Pol II accounts largely for the overall decrease of Pol II recruitment at PSA promoter by flavopiridol treatment (Fig. 4B). This effect was not seen at the promoter of other genes whose expression was not affected by the low concentration of

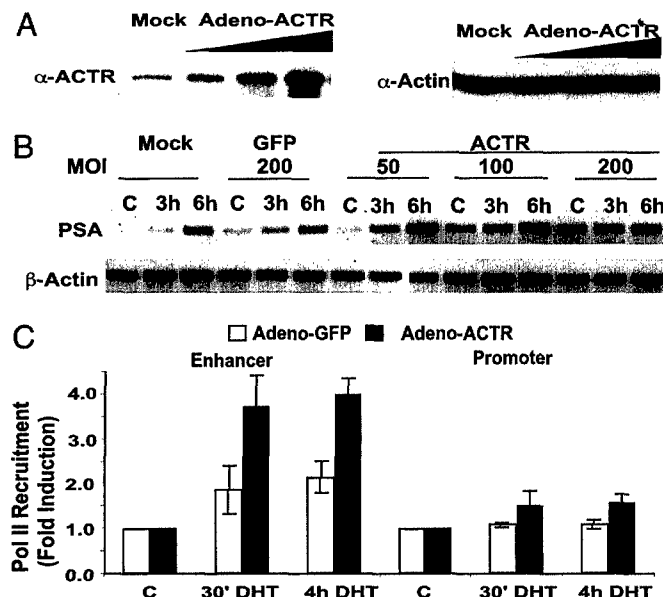


Fig. 5. ACTR mediates PSA transcription by increasing Pol II recruitment at the PSA enhancer. (A) LNCaP cells were infected with adenovirus vector expressing ACTR at different multiplicities of infection (mois) (10, 100, and 200). Whole-cell lysates were prepared 48 h after infection and analyzed by Western blot with antibodies against ACTR (BD Biosciences, San Diego) or β -actin. (B) RT-PCR was performed to measure PSA and β -actin expression in LNCaP cells infected with adeno-GFP or adeno-ACTR vectors at different MOIs and treated with 1 nM DHT for the indicated times before harvest. (C) Elevation of ACTR expression promotes Pol II recruitment to the PSA enhancer. Pol II occupancy at PSA enhancer and promoter was analyzed by ChIP assay with 8WG16 antibody. LNCaP cells were harvested 2 days after infection with adenovirus vectors at moi 100 for either GFP (open bars) or WT ACTR (filled bars) and treated with 1 nM DHT for the indicated times. C, without treatment.

flavopiridol (data not shown). Because blocking transcriptional initiation or elongation would leave more Pol II assembled and stalled at the promoter, as demonstrated for the IL-8 gene (24), the decrease in Pol II occupancy of the PSA promoter by flavopiridol cannot be attributed to the blockade of transcription initiation or elongation at the promoter. Our finding that decreased Pol II occupancy at the promoter is accompanied by its simultaneously increased presence at the enhancer suggests that Pol II recruited at the enhancer of PSA transfers to the promoter and that flavopiridol perturbs this process by inhibiting CTD kinase activity.

ACTR Mediates Androgen Induction of PSA Through Facilitating Pol II Recruitment at Enhancer. Because p160 coactivators ACTR and TIF2 are recruited primarily at the enhancer of PSA (Fig. 1), we decided to test the hypothesis that p160 coactivators might play a role in androgen-induced Pol II recruitment at the enhancer by ectopically expressing ACTR in LNCaP cells (Fig. 5A). When LNCaP cells were stimulated with a suboptimal concentration of DHT (1 nM), increasing the level of ACTR significantly enhanced both androgen-dependent and -independent PSA expression (Fig. 5B). Importantly, a marked increase in Pol II recruitment was detected by ChIP assay at the enhancer but not the promoter in cells with elevated level of ACTR (Fig. 5C). These results provide the evidence that p160 coactivators such as ACTR are involved in androgen induction of PSA expression by facilitating Pol II recruitment to the enhancer.

Discussion

Enhancers are discrete DNA elements that mediate the transcriptional activation of DNA binding transcription factors at a

distance from the promoter. It has long been speculated that enhancers may function as an entry site for RNA polymerase to relay the transcriptional activation signals impinged at the enhancer to the nearby promoter (25). By analyzing the occupancy of Pol II as well as AR and its coactivators over the entire PSA regulatory region, we found that androgen induces the recruitment of Pol II preferentially to the enhancer. We then demonstrated that AR and Pol II co-occupy strongly at the enhancer but not at the proximal promoter, thereby defying the explanation by the simple looping model in which the enhancer indirectly contacts Pol II complex assembled at the promoter. With highly focused analysis, Shang *et al.* (26) recently reported the detection of androgen-induced Pol II association with both PSA promoter and enhancer (26). Although their study did not address the molecular nature of such association, their result is consistent with our conclusion that Pol II is directly recruited at the enhancer. More strikingly, using *in vitro* reconstituted nucleosomal PSA enhancer DNA immobilized on the beads, we obtained results showing unequivocally that Pol II can be recruited to the enhancer independent of the promoter and that the Pol II recruitment at the enhancer entirely depends on the presence of DHT and the purified AR, ACTR, and p300 proteins. Further, the CTD kinase inhibitor flavopiridol promoted Pol II occupancy at the enhancer, with a concomitant block of Pol II occupancy at the promoter. Taken together, these results suggest that the recruitment of Pol II at the enhancer and the promoter may be controlled by distinct mechanisms. Recently, using ChIP assay, it was demonstrated that Pol II occupancy at the locus control region of β -globin genes is regulated by an unidentified mechanism that may differ from that at the β -globin promoter (27). Our finding that, on hormone induction, Pol II is recruited at a conventional enhancer suggests that direct Pol II recruitment may represent a general mechanism underlying enhancer function.

Conceptually, transcription factors and their coactivators or Pol II recruited at a distant enhancer could act on the promoter

by either the looping mechanism or tracking along the chromatin (28). Enhancers in mammalian cells are often >5 or 10 kb away from their cognate promoters. Thus, it is less likely that protein-protein contact between enhancer and promoter is achieved simply by looping out the interval DNA. We tend to favor the "facilitated tracking" mechanism for the Pol II complex to interact with the promoter, based on the observations made in this study. Thus, we found that androgen-induced Pol II cross-linking appears to trail over the entire region of the 6-kb PSA upstream sequence. Intriguingly, the pattern of androgen-induced histone hyperacetylation resembles the pattern of Pol II cross-linking (data not shown). It is conceivable that the hyperacetylation of nucleosomal histones might help the transient association of Pol II with chromatin downstream of the enhancer. In this regard, it is tempting to postulate that one of the functions of Pol II CTD phosphorylation might be to facilitate the association of Pol II complex with chromatin remodeling factors.

In an attempt to assess directly the role of p160 coactivators in AR-mediated induction of gene expression, we observed that elevating ACTR level in prostate cancer cells could result in marked increase of both androgen-dependent and -independent PSA expression. Prostate cancer almost inevitably progresses from a hormone-dependent to a hormone-refractory state with elevated levels of PSA. Therefore, a thorough understanding of the mechanism that governs the expression of PSA will shed light on the mechanism underlying the androgen independence of prostate cancer. Recently, aberrant expression of p160 coactivators was identified in multiple human malignancies including prostate cancer (29). It would be of interest to study the involvement of p160 coactivators in the development of prostate cancer androgen independence.

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**ACTR/SRC-3 and Androgen Receptor Control Prostate Cancer Cell Proliferation
and Tumor Growth via Distinct Mechanisms**

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Abstract

Prostate cancer often progresses from androgen-dependent to hormone refractory stage after androgen ablation. Although current evidence strongly supports the notion that androgen receptor plays a pivotal role in different stages of prostate cancer, the underlying molecular mechanism is poorly understood. ACTR (AIB1, pCIP, RAC3, SRC-3 and TRAM1) was initially identified as a member of the p160/SRC coactivator family for the nuclear hormone receptors including the androgen receptor. It is implicated in multiple types of human cancers because of its frequent overexpression and/or amplification in tumor tissues. To determine the function of ACTR and androgen receptor in control of prostate cancer cell proliferation, we acutely knocked down their expression in androgen-dependent (LNCaP) and hormone-refractory (LNCaP derivatives C4-2B and p53-R273H, and CWR22Rv1) prostate cancer cells and CWR22 tumor xenograft. Our results indicate that high levels of ACTR and androgen receptor are required for proliferation of both androgen-dependent and -independent cells, and for tumor growth. Interestingly, knocking down TIF2, another member of the p160/SRC family, appeared not to affect their proliferation, suggesting that members of the p160 coactivators play different roles in control of prostate cancer cell proliferation. Importantly, depletion of ACTR and androgen receptor affected different sets of cell cycle gene expression as well as the same ones (Cyclin A and E2F1). In C4-2B cells, while androgen receptor depletion inhibited the expression of cyclin D1, cyclin B and cdc2, ACTR depletion reduced the expression of cyclin E and cdk2. Results from chromatin immunoprecipitation (ChIP) and reporter gene assays indicated that androgen receptor and ACTR are recruited to different target gene promoters to activate their expression in androgen-independent manner. Unexpectedly, we also observed that both androgen receptor and ACTR controls different cell cycle genes in LNCaP versus C4-2B cells. Together, these findings suggest that androgen receptor and coactivator ACTR may play distinct roles in the transition of prostate cancer to androgen independence by controlling different gene expression programs.

Introduction

Prostate cancer usually starts as an androgen-dependent tumor. The initial androgen ablation therapy can effectively lead to clinical regression of the tumor. However, after the prolonged treatment, tumors often re-grow and become hormone-refractory or androgen-independent. The fact that androgen receptor (AR), a member of the steroid/nuclear receptor family of hormone-regulated transcription factors, is expressed in nearly all stages of prostate cancers, including recurrent ones, argues for the involvement of AR in the androgen independence of prostate cancer. Several mechanisms have been postulated that may contribute to the androgen-independent activation of AR. These include AR mutations and/or overexpression, post-translational modifications such as phosphorylation elicited by growth factor/protein kinase signaling (1-3).

Like other steroid receptors, AR interacts with accessory factors for its transcriptional regulation of target gene expression (4). Among the co-factors identified, members of the p160/SRC family of coactivators have been implicated in prostate cancer. Thus, significant levels of the coactivators were found in the majority of recurrent tumors, and their expression correlates with Gleason score or with increased tumor aggressiveness (5-8). In particular, overexpression of ACTR (also named as AIB1/pCIP/RAC3/SRC-3/TRAM1) associates with high proliferation rate and low apoptotic index (8). The p160 coactivators, which also include SRC-1, and GRIP1/SRC-2/TIF2, share high homology in several functional domains. These include the central receptor interaction domain (RID), and the C-terminal domains responsible for the interaction with p300/CBP and protein arginine methylases such as CARM1. They facilitate the transcriptional regulation likely through modifying histones and remodeling chromatin (9).

Although members of the p160 coactivators all play important physiological roles in the development and function of mammalian reproductive system, the exact function of each member appears different. For instances, GRIP1/TIF2 ablation in mice affects primarily spermiogenesis and placenta development (10). ACTR/SRC-3 knockouts exhibit significant defects in somatic growth, delayed puberty as well as female reproductive functions (11, 12).

Moreover, amplification and/or overexpression of ACTR, but not the other p160s, has been detected in a broad spectrum of malignancies with high frequency, including pancreatic adenocarcinoma, hepatocellular carcinoma, gastric cancers, colorectal carcinoma, and breast cancer (13-17). Its overexpression in breast cancers does not correlate with positive ER status (16). These findings suggest that members of the p160 coactivators may play different roles in cancer, and that aberrant ACTR may act through multiple mechanisms to promote tumorigenesis. In agreement with this notion, it was recently demonstrated that deletion of ACTR did not affect the tumor promotional role of ovarian hormones in mammary tumor formation (18). Ectopic ACTR expression in prostate cancer cells appears to stimulate the Akt signaling independent of hormone (19). We demonstrated that ACTR overexpression could negate the growth inhibitory effect of anti-estrogens through up-regulation of key cell cycle genes in breast cancer cells (20).

To understand the mechanisms through which androgen receptor and the p160 coactivators control prostate cancer proliferation, we examined the effects of down-regulation of AR, ACTR and GRIP1/TIF2 on the proliferation and gene expression of androgen-dependent and androgen-independent prostate cancer cells. Here we report that high level of AR and ACTR but not GRIP1/TIF2 is required for the proliferation of both androgen-dependent and androgen-independent prostate cancer cells, and that a mechanism for which AR and ACTR to affect cell proliferation is through regulation of cell cycle gene expression. Interestingly, we observed that the expression of distinct subsets of genes is under the control of AR and ACTR in androgen-dependent versus androgen-independent cells, which may underscores the mechanistic difference of their functions in control of prostate cancer growth.

Materials and methods

Cell culture and reporter gene assays. LNCap, LNCap-p53-R273H (21), CWR22Rv1 (22) and PC-3 cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Gemini, Woodland, CA). LNCaP-C4-2B cells were obtained from UroCoR (Oklahoma City) and cultured in T-medium (Invitrogen) with 5% FBS. Cells were maintained at 37°C in a humid atmosphere containing 5% CO₂. Reporter gene assays were performed by

transfecting PC-3 cells with firefly luciferase reporter plasmid pGL3-cyclin E, -cyclin A or -cyclin D1 promoter, pCMV- β -gal plasmid for normalization, pcDNA3-hAR, pcD-HCMV-ACTR-HA, and pCMV-E2F1. Cells were cultured in 24-well plate with a hormone-depleted medium (phenol red-free RPMI1640 supplemented with 10% FBS treated twice with charcoal and dextran) for 24 hours prior to the transfection. Forty-eight hours after the transfection, cells were harvested for luciferase assay using a luciferase kit (Promega, Madison, WI) and β -gal assay. All reporter gene assays were performed in triplicate, with the entire experiment repeated at least twice.

RNAi adenovirus. Oligodeoxynucleotides containing sequences for small interfering RNA (siRNA, such as

5' gatccccGGTCTTACCTGCAGTGGTGAAttcaagagaTTCACCACTGCAGGTAAGACcttttgg aaaa, with sequences in capitals targeting ACTR mRNA) were inserted downstream of the human H1 gene promoter of the modified pShuttle plasmid. The sequences targeting other genes are 5'GGATGGAAGTGCAGTTAGG (AR#1), 5'GCACTGCTACTCTTCAGCA (AR#2), 5'GACCTACCGAGGAGCTTTC (AR#3), 5'TCTGTTCCAGAGCGTGCGC (AR#4), and 5'CCATGTGTCAGGCATGCAAGC (TIF2). The resulting pShuttle constructs were used to generate recombinant adenoviruses according to the protocols described previously (20).

Viruses were purified by centrifugation in a CsCl step gradient. Viral titers were determined by end point cytopathic effect assay and/or Adeno-X Rapid Titer kit (BD Biosciences). Equal titers of the viruses were used in the experiments, except indicated otherwise.

Xenografts and cell proliferation assays. The CWR22 prostate tumor xenografts (a gift from Dr. Thomas A. Pretlow, Case Western Reserve University, Cleveland, OH) were regenerated as described previously (23). For adeno-RNAi treatment, approximately 20 million CWR22 tumor cells isolated from an established tumor were resuspended in 1 ml of RPMI1640 supplemented with 10% FBS, and incubated with gentle shaking at room temperature for 45 minutes with either adeno-RNAi-ACTR, adeno-RNAi-GFP at a titer of 20 MOI. Cells were then mixed 1:1 with 50% Matrigel (BD Bioscience), before injected subcutaneously into 4 to 5 weeks-old nude/nude athymic male mice (10 for each adeno-RNAi treatment, Harlan, Indianapolis, IN).

Two days before the injection, the mice received implants of a testosterone pellet (Innovative Research of America, Sarasota, FL) to stimulate the tumor growth. The tumors were measured twice a week using calipers. Tumor volume was calculated as length x width x height x 0.5234. For cell proliferation assays, five thousands of LNCaP cells were seeded in each well of a 96-well plate. Cells were then maintained in the hormone-depleted medium as described above for 24 hrs before infected with equal titers of adeno-RNAi-AR, adeno-RNAi-ACTR, adeno-RNAi-TIF2 or adeno-RNAi-GFP. Twenty-four hours after the infection, cells were stimulated with 1 nm DHT. Cell proliferation was measured every two days by MTS colorimetric assay (Promega). For LNCaP-p53-R273H, LNCaP-C4-2B and CWR22Rv1 cells, twenty-five hundreds of cells were seeded in each well of a 96-well plate. Cells were then maintained in the medium containing 2.5% the hormone-depleted FBS for 24 hrs before the infection. Forty-eight hours after the infection, concentration of the FBS was increased to 5%. Cell proliferation was measured every two days by MTS colorimetric assay.

Western blotting and quantitative RT-PCR analysis. Cells were seeded in 6-well plates, then infected with adeno-RNAi viruses as above, and collected at various days after the infection. Cell lysates were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma). Equal amounts of cell extracts were analyzed by immuno-blotting using antibodies against following proteins: AR (Upstates), ACTR, TIF2, cyclin A2, cyclin B (Becton Dickinson), Cdk2 (M2), Cdc2 (17), cyclin E (HE12), SRC-1 (M34), E2F1 (KH95), cyclin-D1, -D2, -D3 (Santa Cruz) and actin (Sigma). For quantitative reverse transcriptase PCR (RT-PCR) analysis of gene expression, total RNA was isolated with TRIzol reagent (Invitrogen). Three micrograms of total RNA was used for a reverse transcription reaction with Moloney murine leukemia virus RT and oligo(dT)18 primers; cDNAs were diluted 10-fold, and a 5 µl of dilution was used for the PCRs. Gene sequences were amplified in the presence of SYBR Green fluorophore and detected using iCycler real-time PCR equipment (BioRad). Fluorescent values after each elongation step were collected along with a melting curve analysis at the end of the PCR. Fold difference was calculated, as described previously (20).

Chromatin immunoprecipitation (ChIP) assays. ChIP was performed essentially as described previously (24). LNCaP-C4-2B cells were plated in T-medium containing 5% hormone-depleted FBS and maintained for 48 hours before switched to serum-free T-medium for 24 hrs. Cells were then stimulated with 10% hormone-depleted FBS for 24 hrs before harvested for ChIP. Cells that remained in serum-free medium were used as control. After fixation and sonication, the crude chromatin solution was diluted and incubated with specific antibodies overnight at 4⁰C. PCR was performed using 5 µl of the purified ChIP DNA for 28 cycles with promoter-specific primers. The primer sequences for cyclin A2, cyclin E1 and E2F1 are described previously (20). The primer sequences for cyclin D1 are 5'CAGGGGAGTTTTGTTGAAGTTGC (forward) and 5'GCACACATTGAAGTAGGACACCG (reverse).

Results

Adenovirus vector-mediated RNAi effectively and specifically inhibited the expression of androgen receptor and the p160/SRC coactivators in prostate cancer cells. To determine whether and how high levels of AR and the p160/SRC coactivators control prostate cancer cell proliferation, we acutely reduced the expression of AR and the coactivators ACTR or TIF2 in prostate cancer cells by adenovirus-mediated siRNA expression. As shown in Fig. 1A, the AR protein level was dramatically reduced in cells that were infected with Ad-RNAi-AR#2 and #3, while no effects on AR level was observed with Ad-RNAi-ACTR, Ad-RNAi-TIF2, or the control Ad-RNAi-GFP, when compared with cells mock-infected. Ad-RNAi-AR#2 was chosen for the rest of experiments because it reduced AR expression most effectively. The expression levels of ACTR and TIF2 were also inhibited effectively by the Ad-RNAi-vector treatment (Fig. 1B and 1C). Importantly, the effects were specific for the corresponding coactivator, as Ad-RNAi-ACTR showed no effects on the protein level of the other two members of p160 family, TIF2 and SRC-1, and vice versa.

High level of androgen receptor is required for both androgen-dependent and -independent prostate cancer cell proliferation. Most prostate cancer initially depends on androgens for growth and survival, thus the elimination of androgens or preventing their binding to the androgen receptor is the foundation of current medical management. Therefore, it was of interest to determine whether reduction of androgen receptor levels will affect the growth of androgen-dependent prostate cancer cells. LNCaP cells grown in the hormone-depleted medium were treated with two different titers of Ad-RNAi-AR or the control vector, and subsequently stimulated with 1 nm of DHT. Cell proliferation was measured at different days after the treatment by MTT assay. The results in Fig. 2A show that treatment with Ad-RNAi-AR strongly inhibited DHT-stimulated proliferation of LNCaP cells and that the severity of inhibition appeared to correlate with the Ad-RNAi-AR titers used. Importantly, treatment with the same titers of control vector Ad-RNAi-GFP did not significantly affect cell proliferation (compare Ri-GFP curves with that of mock). These results suggest that high level of androgen receptor is required for the proliferation of androgen-dependent prostate cancer cells. Next, we examined whether the high level of androgen receptor is also required for the proliferation of androgen-independent prostate cancer cells using different cell lines. LNCaP-C4 and C4-2B cells were derived through passage of LNCaP tumors in castrated athymic nude mice (C4), and became metastatic to the bone (C4-2B) (22). LNCaP-p53-R273H was established via the selection of LNCaP sub-lines that ectopically express the “gain-of-function” mutation allele of tumor suppressor p53 (21). These cells express AR and members of the p160/SRC coactivators at a level comparable to their parental LNCaP cells (unpublished data). The proliferation of these LNCaP derivatives in hormone-depleted medium was greatly inhibited by Ad-AR-RNAi vector treatment (Fig. 2B and 2C). Remarkably, knocking down AR in LNCaP-C4-2B cells caused a precipitant decrease in the number of live cells at 6 days after the vector treatment, suggesting that high level of AR is also required for the survival of these cells. CWR22Rv1 cells were derived from the hormone-refractory tumor of the CWR22 xenograft, and express two forms of AR protein (25). To effectively reduce both forms of AR expression, we generated an Ad-RNAi-AR vector (#2+#3) that expresses two siRNAs targeting different regions of the AR mRNA in CWR22Rv1 cells (Fig 2D). Indeed, Ad-RNAi-AR-#2+3 showed higher potency than Ad-RNAi-AR-#2 in silencing both forms of AR, and strongly inhibited the androgen-independent

proliferation of CWR22Rv1 cells in a dose-dependent manner (Fig. 2D). Together, these results suggest that high level of androgen receptor is required for the proliferation of both androgen-dependent and -independent prostate cancer cells that are androgen receptor-positive.

High level of coactivator ACTR, but not TIF2, is required for both androgen-dependent and androgen-independent prostate cancer cell proliferation. We took the same approach as described above to determine the effect of knocking down the coactivator expression on the proliferation of the four different prostate cancer cell lines. The result shown in Fig. 3A demonstrated that partial reduction of ACTR level was sufficient to inhibit DHT-stimulated LNCaP cell proliferation. Interestingly, similar knocking-down of ACTR in the androgen-independent cancer cells showed much more striking effect in inhibiting the androgen-independent proliferation of the LNCaP derivatives (Fig. 3B and 3C) as well as CWR22Rv1 (Fig. 3D). These results suggest that, like AR, high level of ACTR is required for both androgen-dependent and independent prostate cancer cell growth. Surprisingly, under the same condition, knocking down TIF2 showed no significantly negative effect on the proliferation of either LNCaP or the other three androgen-independent cells. In fact, TIF2 reduction in LNCaP-C4-2B and LNCaP-p53-R273H cells appeared to enhance slightly their androgen-independent proliferation. These results suggest that TIF2 may play a role distinct from that of ACTR in control of prostate cancer cell proliferation.

Silencing ACTR expression suppresses tumor formation in mouse xenografts of prostate cancer. The crucial role played by ACTR in control of prostate cancer cell proliferation prompted us to examine the effect of knocking down ACTR on tumor formation. Cells freshly isolated from the CWR22 tumor xenografts were infected with Ad-RNAi-ACTR and the control vector Ad-RNAi-GFP, mixed with Matrigel and injected into athymic nude mice that were implanted with testosterone pellet to stimulate tumor growth. Similar to the mice injected with non-treated CWR22 tumor cells, mice injected with cells treated with Ad-RNAi-GFP developed tangible tumors around two weeks after injection; the developed tumors grew in a similar pace (Fig. 3E), indicating that treatment with the adeno-RNAi vector alone does not affect tumor growth. In contrast, the mice that received the same number of cells but treated with Ad-RNAi-

ACTR did not develop any tangible tumor four weeks after the injection when the control mice had to be sacrificed due to the excessive tumor burden. These results suggest that high level of ACTR is required for prostate tumor growth.

Androgen receptor and ACTR are required for the expression of distinct cell cycle genes.

To address the molecular mechanism underlying the crucial role of AR and ACTR in control of prostate cancer cell proliferation, we first examined the effect of knocking down AR and ACTR on key cell cycle gene expression. Thus, LNCaP and LNCaP-C4-2B cells were treated with the Ad-RNAi vectors as before. Different days after the treatment, cells were analyzed for the expression of the key cell cycle regulatory proteins by Western blotting (Fig 4) and quantitative RT-PCR (Fig 5 and data not shown). As shown in Fig 4A and 4B, knocking down AR or ACTR in LNCaP cells resulted in a decrease of E2F1 and cyclin D1 protein level. Interestingly, knocking down AR, but not ACTR, significantly inhibited the expression of cdc2 and cdk2 proteins. Conversely, knocking down ACTR, but not AR, decreased the expression of cyclin A and cyclin B. These effects can be observed at 2 days after the Ad-RNAi treatment when AR and ACTR protein levels were significantly reduced.

When we extended the analysis to LNCaP-C4-2B, we observed that the effect on cell cycle gene expression is not only distinct between AR and ACTR depletion, it is also different between LNCaP and its derivative C4-2B. Thus, in C4-2B cells, while both AR and ACTR reduction led to decreased expression of E2F1 and cyclin A, knocking down AR, but not ACTR, also significantly inhibited the expression of cyclin B, cdc2 and cyclin D1 proteins; and knocking down ACTR, but not AR, also decreased the expression of cyclin E and cdk2. Remarkably, the effect of AR knockdown on cyclin A and cyclin B and the effect of ACTR knockdown on cyclin E and cdk2 in the C4-2B cells was not observed in their parental LNCaP cells. On the other hand, the effect in LNCaP cells of AR knockdown on cdk2 and ACTR knockdown on cyclin B and cyclin D1 was not seen in C4-2B cells. Furthermore, the results of quantitative RT-PCR analysis with C4-2B cells shown in Fig.5 indicated that the differential effects of AR and ACTR depletion is largely mediated at the mRNA level for cyclin E, cyclin B, cdc2 and cdk2, as >2-fold decrease of their transcripts can be observed with cells treated with the corresponding Ad-

RNAi vectors. These results cannot be attributed to a possibility of different extent of reduction in AR or ACTR level, or a potential off-target effect by the RNAi, in the two closely related cells. Rather, the data imply that AR or ACTR controls the proliferation of LNCaP and C4-2B through different mechanisms. Since C4-2B was derived from LNCaP as LNCaP xenograft tumor evolved from androgen-dependent state to androgen-independent, these results also imply that the progression of prostate cancer to androgen-independency may involve re-programming of target genes for the crucial regulatory proteins such as AR and ACTR.

Androgen receptor and the coactivator ACTR are selectively recruited to the promoters of different target genes and activate their expression. To determine whether the distinct subsets of cell cycle genes affected by AR and ACTR depletion in C4-2B cells are under the direct regulation by AR or ACTR, we first examined whether the promoters or the distal regulatory sequences of the subset of genes are occupied by AR and/or ACTR when their expression is induced in response to hormone-independent growth stimulation by serum. Thus, cells with or without serum stimulation were harvested for ChIP assay with normal IgG, or AR and ACTR antibodies. The immunoprecipitates with normal IgG yielded very low PCR signals irrespective of the serum treatment and therefore were used as negative controls. Results in Fig.6A showed that, on the cyclin A regulatory sequences, the occupancy by both AR and ACTR were significantly increased (4-5 fold) at the distal upstream region (around -3642 and -3386), but not at the proximal promoter region (around -386 and -69) in response to the serum stimulation. Similar induced recruitment of AR and ACTR were observed at the promoter of E2F1 (Fig. 6B). Strikingly, ACTR but not AR was recruited to the promoters of cyclin E (Fig. 6C) and cdk2 (data not shown). On the contrary, AR but not ACTR was recruited to the promoter of cyclin D1 (Fig. 6D). These observations suggest that AR and ACTR are selectively recruited to the target genes upon growth stimulation by serum.

To further examine the androgen-independent function of AR and ACTR on the target gene promoters, we performed reporter gene assays with the androgen-independent prostate cancer cell PC-3 which lacks AR, and reporter constructs having the luciferase gene directly linked to the promoter sequence of cyclin A, cyclin E or cyclin D1. Each of the promoter sequence used

contains the region where AR and/or ACTR recruitment were observed in the ChIP assays. As shown in Fig 7A, expression of AR alone slightly enhanced the reporter activity of cyclin A promoter, but simultaneous expression AR and ACTR significantly increased the promoter activity, suggesting that AR and ACTR can synergically activate cyclin A promoter under this condition. In contrast, on the cyclin E promoter reporter, expression of AR alone, or in combination with ACTR did not have any significant effect on the promoter activity, although expression of ACTR and E2F1 dramatically increased the activity (Fig 7B). Interestingly, under the same condition, only AR was able to activate the cyclin D1 promoter activity (Fig. 7C). Taken together with the data from the ChIP assay, these results suggest that AR and ACTR, individually or in combination, can directly regulate cycle gene expression in an androgen-independent manner.

Discussion

Consistent with the notion that AR plays a pivotal role in all stages of prostate cancer and that aberrant p160/SRC coactivators may be involved in the androgen-independence of prostate cancer, we demonstrated in this study that AR and ACTR play a crucial role in the proliferation of androgen-dependent and -independent prostate cancer cells that were derived from different tumors and sustained different genetic/epigenetic alterations. These observations are consistent with the recent report that knocking down AR coactivators inhibits the proliferation of AR-positive or AR-negative prostate cancer cells (7, 8, 26, 27). Importantly, our study also revealed that one of the mechanisms underlying the crucial role of AR and ACTR appears to be their direct involvement in control of cell cycle gene expression. At least in the case of cyclin A, AR and ACTR may act together to stimulate cyclin A expression in an androgen-independent manner. More significantly, however, we found that, in both androgen-dependent and -independent cells, AR and ACTR controls the expression of distinct subsets of key cell cycle genes, and that the distinction can be attributed, at least in part, to their selective occupancy on the corresponding target gene promoters. Given our previous observation that ACTR can interact with E2F1 to control cell cycle gene expression in breast cancer cells (20), it is tempting to

speculate that the ability of ACTR to control the expression of cyclin E and cdk2 may involve its association with member(s) of the E2F family. Further study will be needed to identify the exact mediator(s) of ACTR in control of cell cycle gene expression in prostate cancer cells.

The function of androgen-activated AR in mediating cell proliferation is likely through its control of key cell cycle gene expression (28-32). Although a number of genes with expression stimulated by androgen have been reported, the proliferation-promoting genes that are directly regulated by AR still remain being identified, and so is the nature of the regulation. One possibility is that AR is recruited to these regulatory genes through interaction with other transcription factors. It has been documented that estrogen receptor and thyroid hormone receptor can be recruited to cyclin D1 promoter through association with AP1 or CREB (33, 34). Beta-catenin, which can directly control cyclin D1 expression, have been demonstrated to interact with AR, and such interaction can modulate growth factor-stimulated AR transcriptional activity (28, 35-38). On the other hand, cyclin D1 has been shown to interact with AR to modulate its hormone-stimulated transcriptional activity (39, 40); such interaction may serve as a negative feedback mechanism in androgen-dependent cell proliferation. Our results that AR targets cyclin D1 promoter to stimulate cyclin D1 expression in androgen-independent cells suggest a shift from the feedback loop to a more direct action by AR in the cancer progression. Consistent with this, examination of cyclin D1 level in prostate cancer revealed that its overexpression correlates with tumor cell proliferation index and bone metastasis (41). Moreover, cyclin D1 ectopic expression can confer hormone refractory status to LNCaP cells (42).

It has been postulated that, in androgen-independent cells, when AR is activated, it would act together with the coactivators along a pathway that is similar to that of androgen-activated AR. However, the unexpected observation from this study that both AR and ACTR controls different cell cycle genes in the two closely related cell lines LNCaP and C4-2B suggests a more complex involvement of gene expression control mechanism during the transition to androgen independence. At least two related events might occur during the transition, which may account for our observation of differential gene regulations by both AR and ACTR in the two cell lines.

One is that during the adaptation to androgen deprivation, cells reprogram their gene expression regulatory circuitry such as shifting from an androgen-dependent one to a growth factor-directed one, thus altering the levels of key cell survival and cell cycle proteins and/or their activities. Indeed, this kind of gene expression change has been observed during androgen withdraw and tumor recurrence (43-46). The other is that crucial regulatory factors such as AR and ACTR adopt different functions such as engaging in novel protein-protein interactions, in response to changes in the regulatory circuitry. This hypothesis is in agreement with the recent findings that AR may control androgen-independent PSA expression in C4-2B cells without direct occupying the canonical AR binding sites (47), and that coactivator p300 may bypass AR in mediating androgen-independent stimulation of PSA expression (48). Further understanding of these molecular events would provide valuable insights to the progression of prostate cancer.

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Figure legends

Figure 1. Adenovirus-mediated siRNA effectively and specifically knock down the expression of androgen receptor and the p160 coactivators, ACTR and TIF2. *A*, LNCaP cells were plated in hormone-depleted medium for 24 hrs before infected with recombinant adenoviruses (10MOI) that express siRNAs specifically silencing the expression of indicated genes. Cells were then stimulated with 1 nm DHT and harvested at 3 days after infection for Western analysis. *B-C*, LNCaP-C4-2B cells were plated in T-medium with 2.5% hormone-depleted FBS for 24 hrs prior to infection with the RNAi adenoviruses as indicated. Forty-eight hours after the infection, the concentration of serum was increased to 5%. Cells were harvested at 3, 4, and 5 days after the infection for Western Blotting.

Figure 2. Down-regulation of androgen receptor expression inhibits both androgen-dependent and -independent prostate cancer cell proliferation. *A*, LNCaP cells were plated on 96-well plates with hormone-depleted medium 24 hrs before infected with 5 or 10 MOI of Adeno-RNAi-AR#2 (Ri-AR) or Ad-RNAi-GFP (Ri-GFP) or mock infected. Twenty-four hours after the infection, cells were stimulated with 1 nm DHT. Different days after infection, cell proliferation was measured by the colorimetric MTT assay. *B-C*, LNCaP-C4-2B and LNCaP-p53-R273H cells were plated on 96-well plates in their corresponding medium supplemented with 2.5%

hormone-depleted FBS 24 hrs before infected with 10 or 20 MOI adeno-RNAi, or mock infected. Forty-eight hours after the infection, serum concentration in the medium was raised to 5%. Cell proliferation was measured by the MTT assay. *D*, Expression of androgen receptor in the androgen-independent cell line CWR22Rv1 was knocked down by simultaneously expressing siRNAs targeting two different regions of AR mRNA, as indicated by the arrows (left panel). CWR22Rv1 cells were treated 40 or 80 MOI adeno-vectors, and harvested for MTT assay at indicated days, as in B-C (right panel).

Figure 3. Knocking down ACTR inhibits androgen-dependent and independent prostate cancer cell proliferation and tumor formation. *A-D*, Cells were plated, infected with 20 MOI adeno-RNAi, and treated as in Figure 2A. Cell proliferation was measured by MTS assay. *E*, Freshly isolated CWR22 tumor cells were infected with either adeno-RNAi-ACTR or adeno-RNAi-GFP at MOI 20, mixed with Matrigel, and then injected into nu/nu athymic male mice, which received implants of a testosterone pellet before the injection. The growth rates of tumor xenografts were measured using calipers.

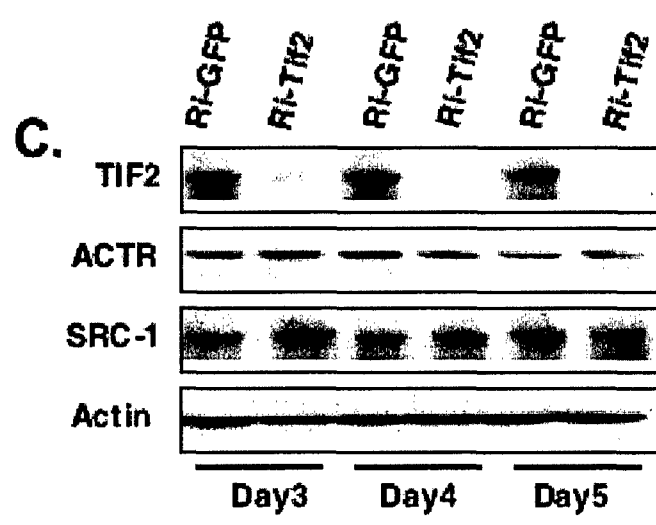
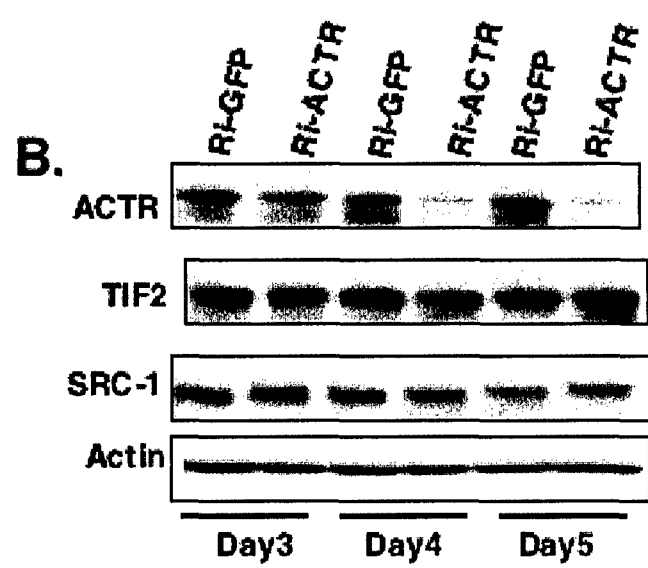
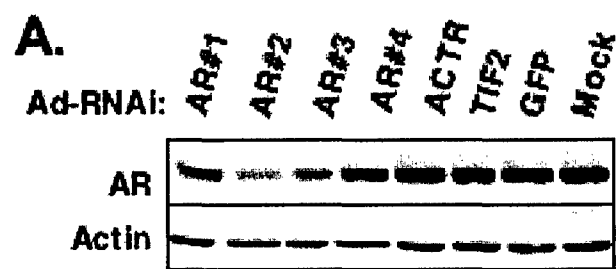
Figure 4. Depletion of androgen receptor or ACTR affects expression of distinct groups of cell cycle regulatory proteins. Cells in 6-well plates were infected with indicated adeno-RNAi viruses as described in Figure 2. Cells were harvested at different days after infection for Western analysis of protein expression.

Figure 5. Depletion of AR or ACTR affects the expression of cell cycle regulatory genes at mRNA level. LNCaP-C4-2B cells were plated in a 6-well plate with 2.5% cds-T medium for 24 hours prior to infection with 5 TCID₅₀ of Ad-RNAi-AR, Ad-RNAi-ACTR or Ad-RNAi-GFP viruses. Forty-eight hours after the infection, cells were collected for preparation of mRNA. The mRNA expression levels in the infected cells were determined by real-time PCR. Fold decrease was determined by comparing the relative level of mRNAs from Ad-GFP-RNAi infected cells with that from Ad-RNAi-AR or Ad-RNAi-ACTR infected cells. Cells were harvested at 2.5, 3 or 3.5 days after the infection. Details of procedure and calculation of difference are described in Materials and Methods.

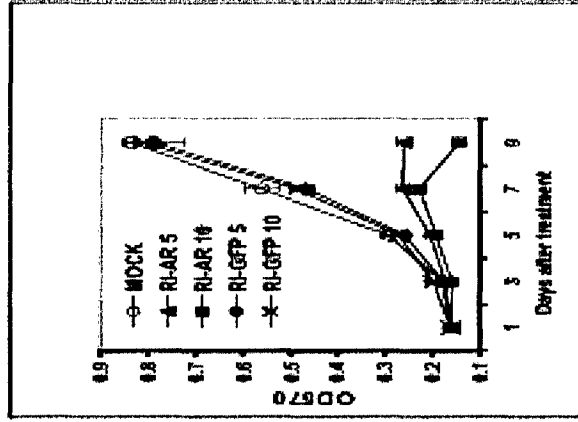
Figure 6. Androgen receptor and coactivator ACTR are selectively recruited to the promoters of cell cycle genes. The recruitment of AR and ACTR to the promoters of cell cycle gene cyclin A2 (A), E2F1(B), cyclin E1 (C) and cyclin D1 (D) was analyzed by ChIP assay.

LNCaP-C4-2B cells were plated in T medium supplemented with 5% hormone-depleted FBS for forty-eight hours. Medium was then replaced with serum-free T-medium for 24 hrs before switched to T-medium with 10% hormone-depleted FBS for another 24 hrs of stimulation. Cells that were remained in serum-free medium for 24 hrs were used as a control. Equal number of cells from serum-free or the FBS supplemented medium were used for the ChIP assay. Cell lysates were divided equally and immunoprecipitated with anti-AR, anti-ACTR or the normal IgG antibody as a control. Fractions (5%) of total soluble chromatin preparations were taken before immunoprecipitation and used as input; the DNA was isolated in the same way as for the ChIP assay. Usually 1/10 of the DNA isolated from the input and the ChIP samples was used as template for generation of the PCR products. Regions of genomic sequence analyzed by PCR analysis are indicated on the schematic of each gene promoter.

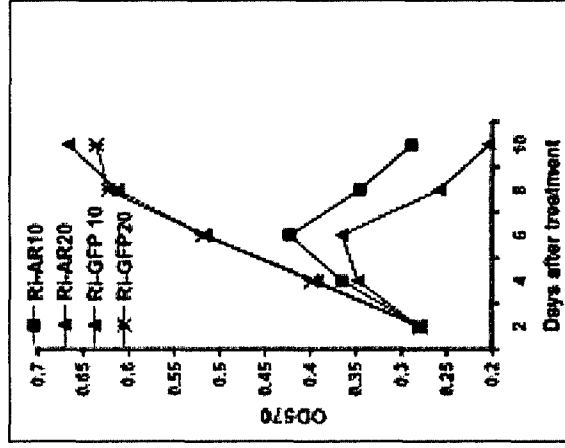
Figure 7. Androgen receptor and coactivator ACTR selectively activate cell cycle gene expression. Ability of androgen receptor and coactivator ACTR to transactivate cell cycle gene promoters was examined using the reporter gene assay. PC-3 cells in hormone-depleted medium were transiently transfected with human cyclin A2 (A), cyclin E1 (B) and cyclin D1 (C) promoters linked to the luciferase gene, along with plasmids expressing AR, ACTR, and E2F1, as indicated. Forty-eight hours after the transfection, cells were harvested for the luciferase assay.



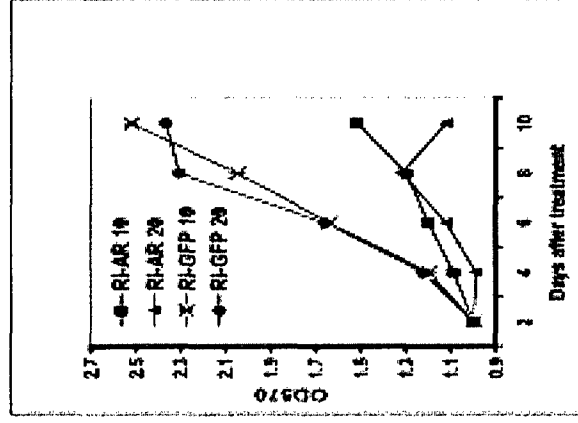
A. LNCaP



B. LNCaP-C4-2B



C. LNCaP-p53-R273H



D. mRNA: RNAi#3



Proteins: (Q)n (G)n DBD LBD C

Ad-RNAi: AR#2 AR#2+3 AR#2 AR#2+3

AR < 114 80

Actin

1 2 3 4 5 6 7 8 9 10

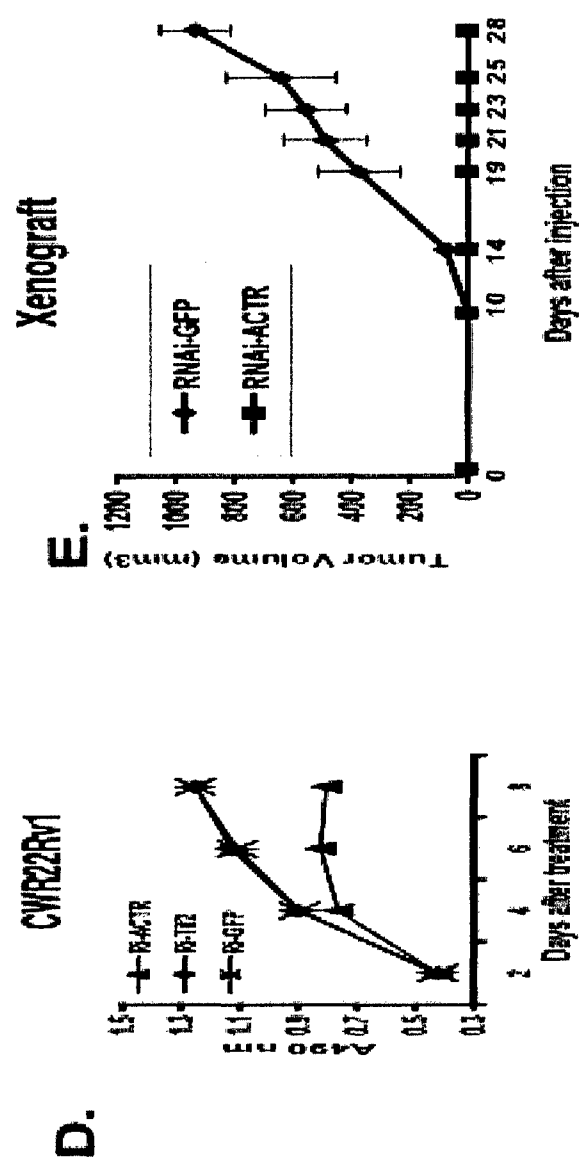
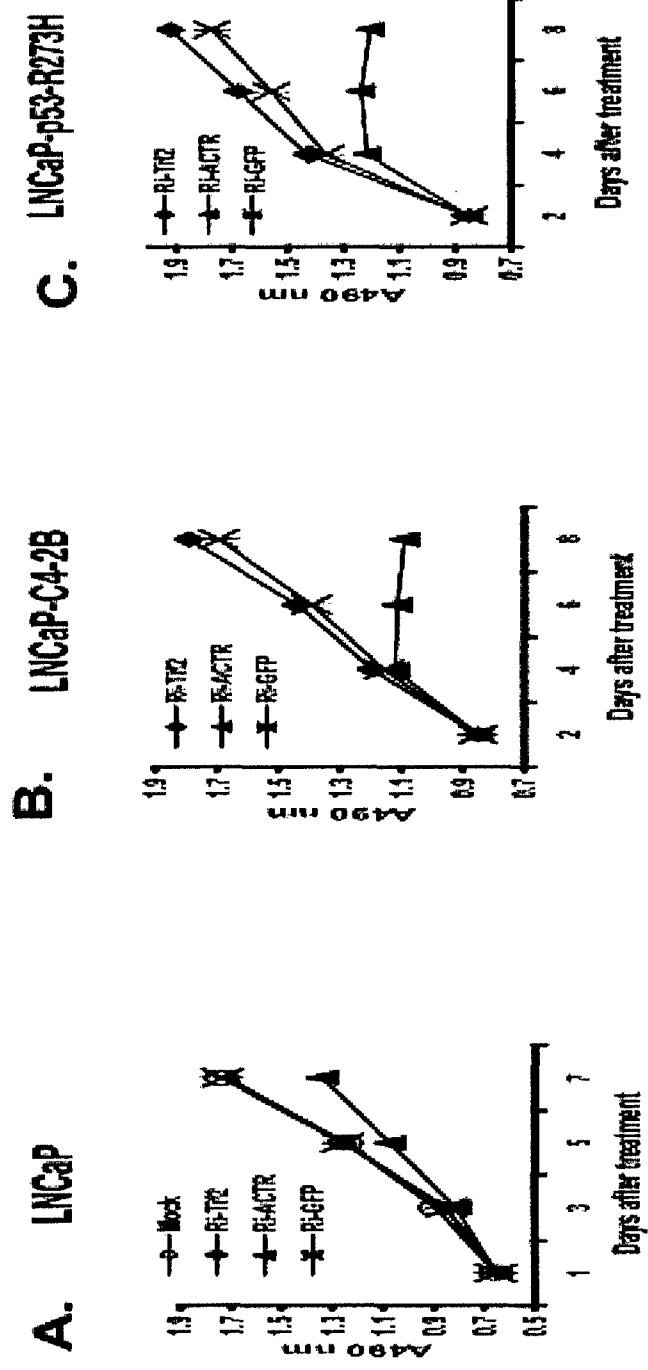
3 days 5 days

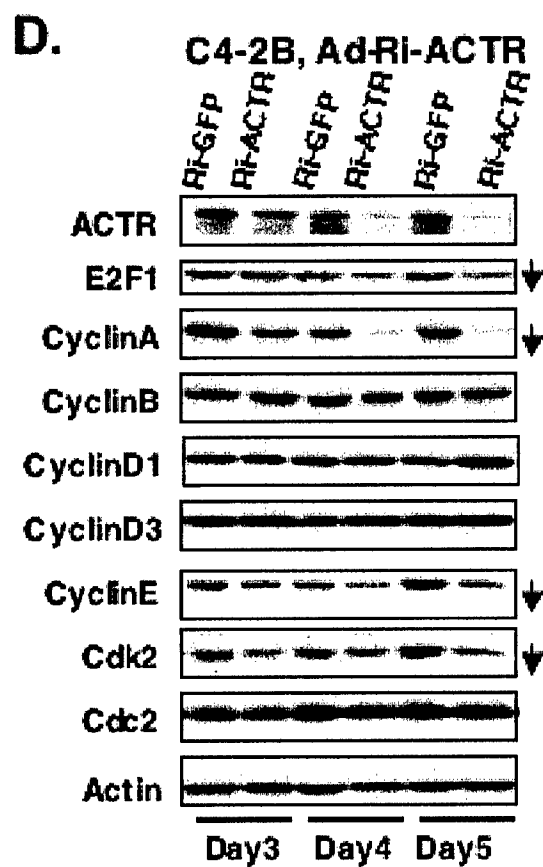
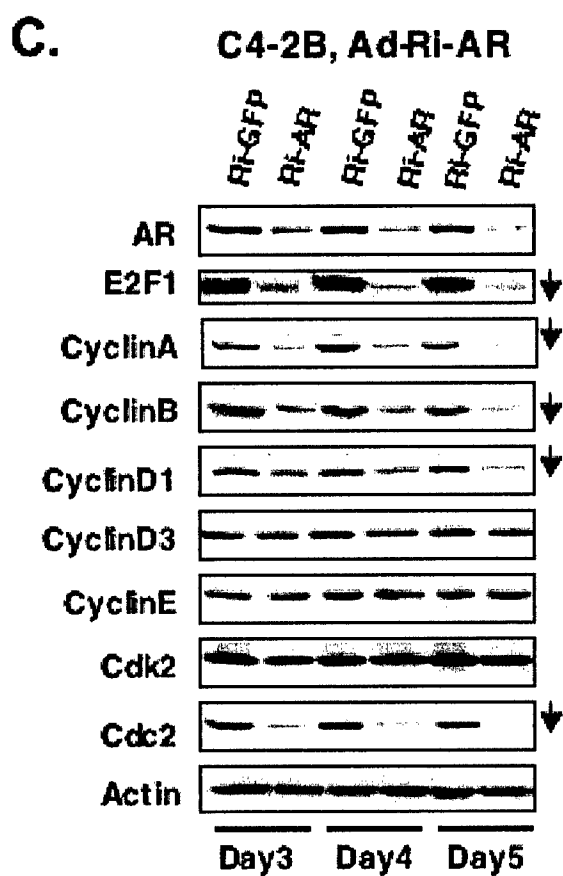
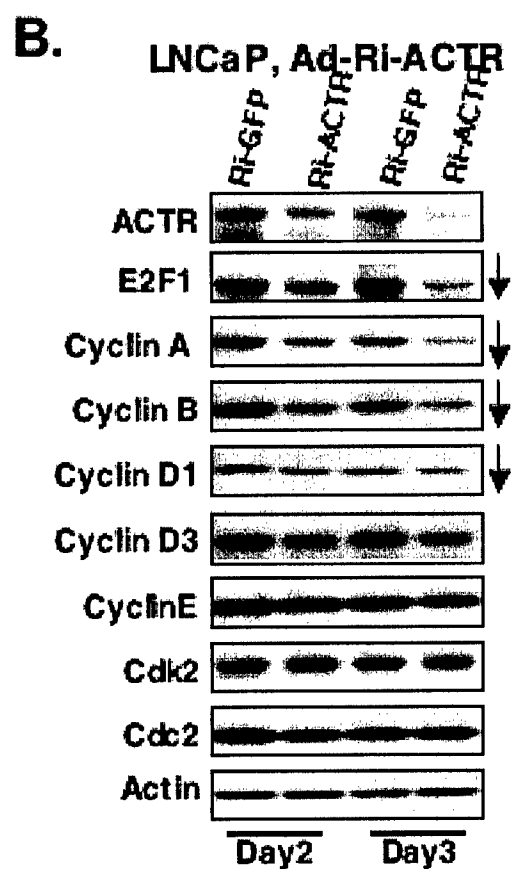
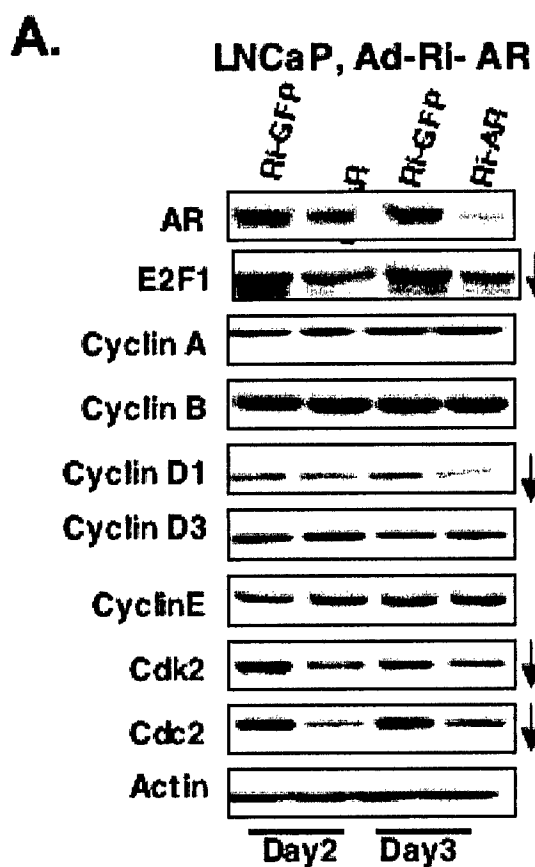
AR#2 AR#2+3

AR#2 AR#2+3

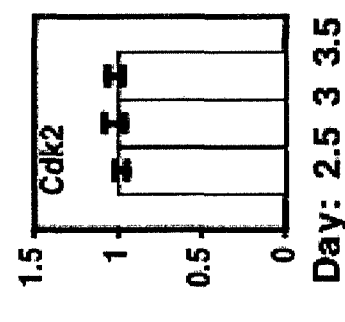
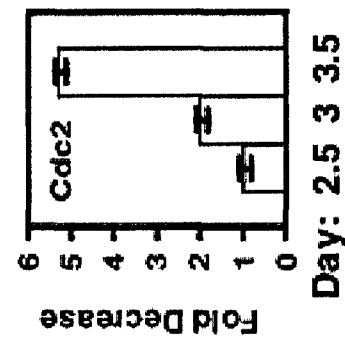
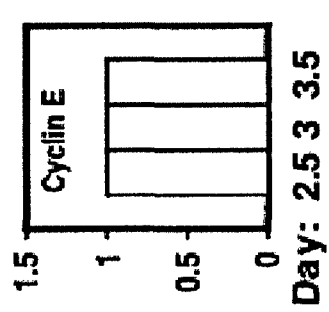
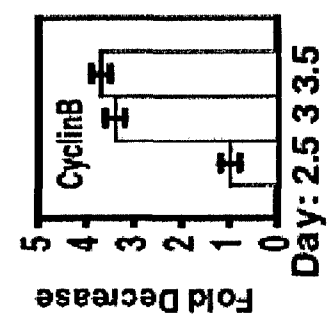
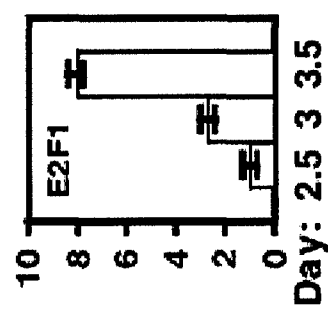
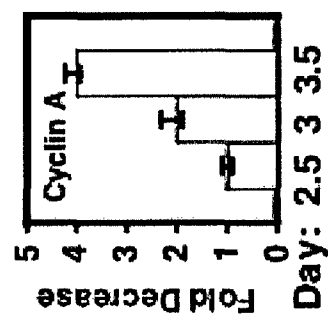
AR#2 AR#2+3

AR#2 AR#2+3

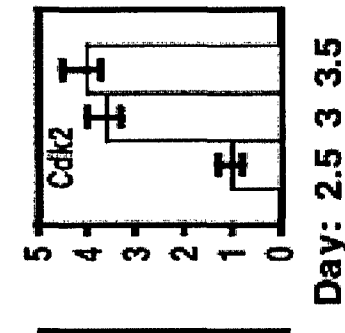
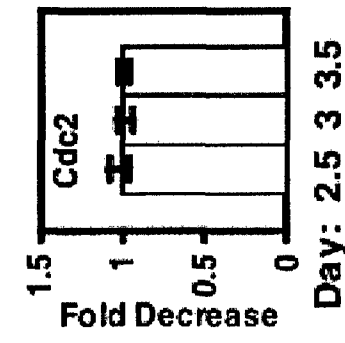
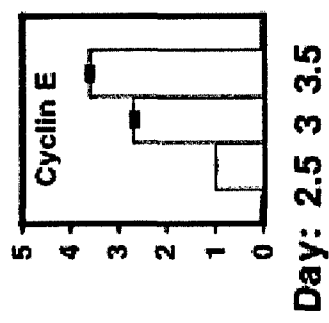
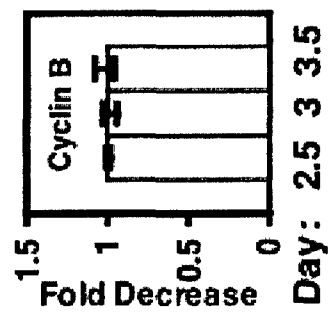
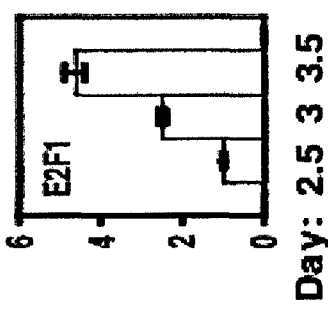
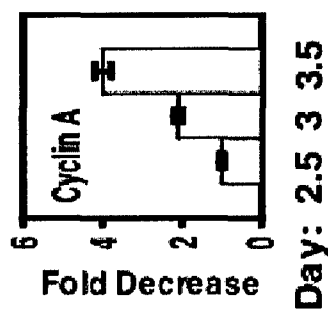


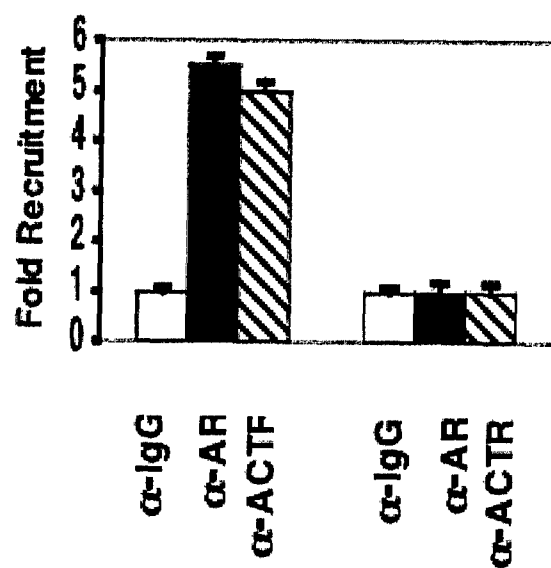
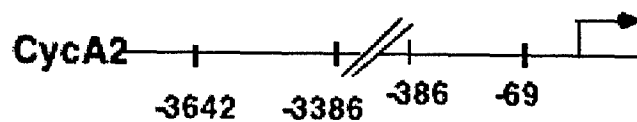
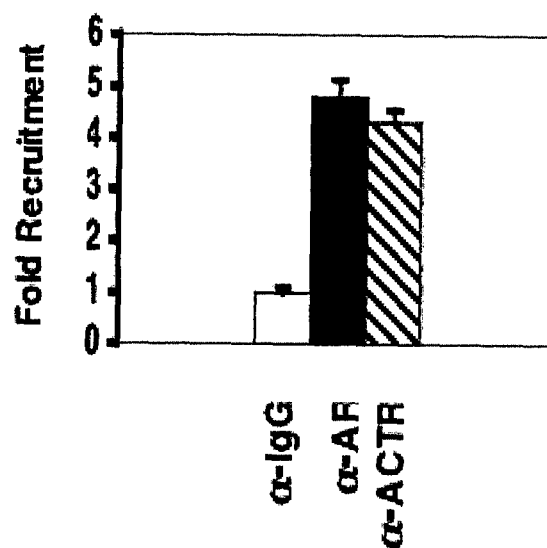
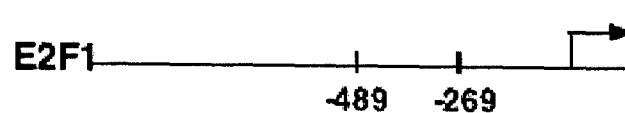
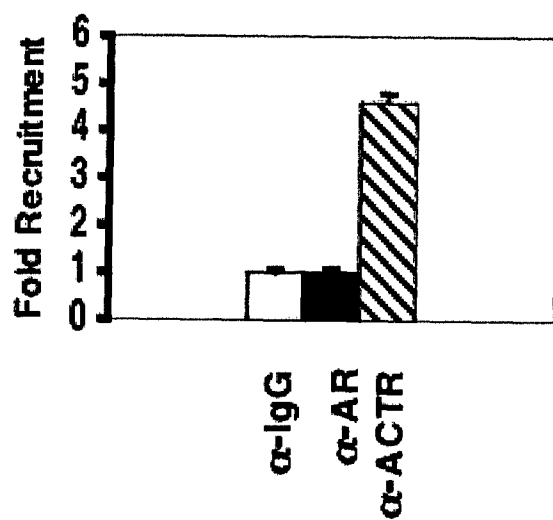
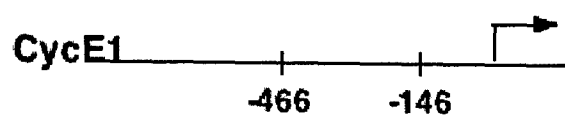


A. C4-2B, Ad-RNAI-AR



B. C4-2B, Ad-RNAI-ACTR



A.**B.****C.****D.**